PROCEEDINGS OF THE AUSTRALIAN PHYSIOLOGICAL AND PHARMACOLOGICAL SOCIETY

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AUSTRALIAN PHYSIOLOGICAL AND PHARMACOLOGICAL SOCIETY INC.

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Communications of the Sixty-Eighth Meeting of the Society at RMIT University

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Submission of Abstracts

There will be no Meeting of the Society in 2001, when the IUPS Meeting is to be held in Christchurch from August 26th to September 1st (see inside front cover, which has the Congress Website address).

In 2002 the APPS Meeting will be part of the ASMR Health & Medical Research Congress. ASMR have invited over 40 Australian societies to participate in a joint congress in 2002. At present the dates and venue have yet to be determined: it likely to be held in late November at the Melbourne Exhibition and Conference Centre

Members should consult the APPS Website www.apps.org.au for further information.

The National Secretary's email address is rick.lang@med.monash.edu.au .

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STUDENT PRIZES

At each of the Society's meetings, prizes are awarded for the most outstanding presentations by student members (including applicants for student membership). Normally, two awards are made in each of the categories "Oral" and "Poster". The presentations are judged by a panel of senior physiologists and pharmacologists.

The Society acknowledges the generous support of the joint sponsors of these awards, **Blackwell Scientific Publications**, (publishers of *Clinical and Experimental Pharmacology and Physiology*), and **SDR Clinical Technology.**

The winners of the awards at the Sixty-seventh Meeting in Newcastle in September 1999 are listed below. The P numbers refer to the abstracts in Issue 30(2) of the *Proceedings*.

ORAL PRESENTATION

***CEPP* Prize**	**SDR Clinical Technology Prize**
Damien Angus	Edoardo Aromatis
Physiology	Physiology
Melbourne	Adelaide
51P	**57P**

POSTER PRESENTATION

SDR Clinical Technology Prize	***CEPP* Prize**
Rebecca Haddock	Glenis Crane
Neuroscience	Human Physiology
JCSMR	Flinders
77P	**89P**

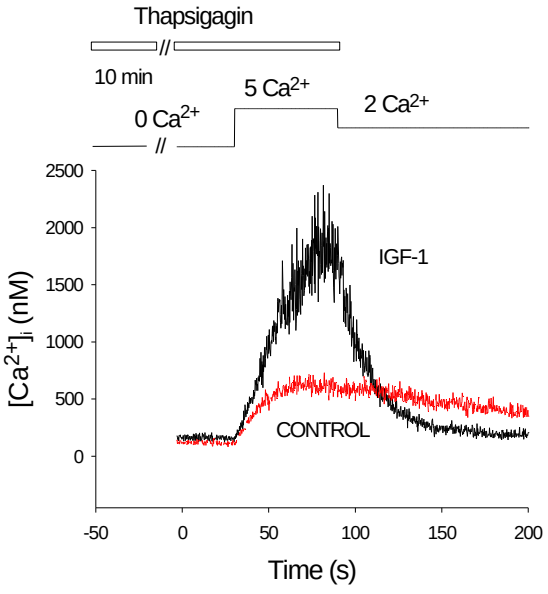
IGF-1 ENHANCES STORE-OPERATED Ca^{+} CHANNEL ACTIVITY IN C2C12 MYOBLAST

Yue-Kun Ju[*], *Ming-Jie Wu*[†], *Robert M. Graham*[†] *and David G. Allen*[*], [*]*Department of Physiology, University of Sydney, NSW 2006 and* [†]*Molecular Cardiology Unit, Victor Chang Cardiac Research Institute, Darlinghurst 2010.*

Skeletal muscle hypertrophy in C2C12 myoblasts stably transfected with insulin-like growth factor-1 (IGF-1-C2C12 myoblasts) is associated with increased $[Ca^{2+}]_i$ and the activation of the Ca^{2+}-calmodulin-dependent phosphatase, calcineurin (Semsarian *et al*., 1999). We have found that increased $[Ca^{2+}]_i$ was due to enhanced Ca^{2+} entry that was resistant to L-type Ca^{2+} channel blockade. In the present study, we try to identify a pathway for Ca^{2+} entry in C2C12 myoblast cell line.

It has been reported that a surface membrane protein, CD20, could function as a calcium-permeable cation channel (Kanzaki *et al.*, 1997). IGF-1 enhanced CD20 channel activity allowing increased changes in $[Ca^{2+}]_i$ in response to changes in $[Ca^{2+}]_o$. To investigate whether Ca^{2+} entry is also through a "CD20" channel in C2C12 myoblasts, we first checked the expression of CD20 in these cells. CD20-like protein with MW of 33 to 37 KDa was found by Western analysis. Phosphate metabolic labelling study indicates there were more phosphorylated CD20 proteins in IGF-1-C2C12 than control. Northern blotting and RT-PCR also further confirmed the existence of CD20 mRNA. However, sudden increase of extracellular $[Ca^{2+}]_o$ from 0 mM to 10 mM, only caused a small change in $[Ca^{2+}]_i$. Therefore, there was no evidence of conventional CD20 channel acted as Ca^{2+} entry route in C2C12 myoblasts.

However, if cells were in Ca^{2+} free solution for a longer period time, the increase of $[Ca^{2+}]_o$ did cause a substantially increase of $[Ca^{2+}]_i$, especially in IGF-C2C12 myoblasts. It is known that a type of plasma-membrane Ca^{2+} channel called as store operated Ca^{2+} channel (SOC), opens in response to a decrease in the concentration of Ca^{2+} in intracellular Ca^{2+} stores. In non-excitable cells SOC play an important role in various cell functions, included cell proliferation and differentiation (Barritt, 1999). To test whether C2C12 myoblasts had SOC activity, we studied the $[Ca^{2+}]_i$ changes in response to sudden increasing $[Ca^{2+}]_o$ after empting intracellular Ca^{2+} stores. Following the application of thapsigargin, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, the increase $[Ca^{2+}]_o$ from 0 mM to 5 mM indeed induced a much larger $[Ca^{2+}]_i$ rise. In IGF-1-C2C12 cells the increasing of $[Ca^{2+}]_i$ was greater than the control (see Figure). The results suggested that SOC activities were greatly enhanced by IGF -1.



Surprisingly, we found that CD20 antibody blocked SOC related Ca^{2+} entry. SOC activity was also greatly increased in C2C12 cell line transfacted with CD20. Since the structure of SOC is uncertain (Barritt, 1999), our results suggested that CD20 could be a new candidate for SOC protein.

Barritt, G.J. (1999) Biochemical Journal, 337, 153-169.

Kanzaki, M., Nie, L., Shibata, H. & Kojima, I. (1997) Journal of Biological Chemistry, 272, 4964-4969.

Semsarian, C. Wu, M-J., Ju, Y-K., Marciniec, T., Yeoh, T., Allen, D.G., Harvey, R.P. & Graham, R.M. (1999) Nature, 400, 576-581.

Supported by the NH&MRC.

Ca^{2+}- AND Sr^{2+}-ACTIVATION PROFILES OF FIBRES FROM A FAST-TWITCH MUSCLE FROM THE HINDLIMBS OF AN AUSTRALIAN MARSUPIAL, THE TAMMAR WALLABY (*Macropus eugenii*), DURING DEVELOPMENT

Jan M. West, Anthony R. Luff, David W. Walker and Marilyn Renfree, Department of Physiology, Monash University, Clayton 3800 Victoria Australia.*

Tammar wallabies are born weighing approximately 0.4 g and grow to a final adult size of 5-8 kg. After birth, the young makes an arduous journey to the pouch and once secured to a teat remains permanently attached to it for approximately 100 days. During this first 100-150 days the young grows slowly, reaching a body weight of around 100 g. A growth spurt is observed between 150 and 220 days. The young can stand unaided by day 160, make their first excursion from the pouch at day 190, and leave the pouch permanently by day 250. The aim of this study was to determine the Ca^{2+}- and Sr^{2+}-activation properties of single muscle fibres from a fast- (deep digit flexor muscle (DDF)) and a slow-twitch (soleus) muscle from young adult animals and from the fast-twitch muscle from pouch young of different ages.

Animals were humanely killed and muscle was dissected from young adult animals and from pouch young at 64, 106 and 131 days. The muscles were chemically skinned in a relaxing solution containing glycerol (50% v/v) and stored at -20ºC. Single fibres were isolated under paraffin oil, attached to a force recording apparatus and isometric force measured. Force development was induced by placing the fibres in EGTA buffered solutions containing different concentrations of Ca^{2+} and Sr^{2+}. Sr^{2+} has proven a useful tool in the identification of different mammalian muscle fibre types (West *et al*., 1999). In general, fast-twitch fibres have a greatly different sensitivity to Ca^{2+} and Sr^{2+} whereas slow-twitch fibres have a similar sensitivity to these divalent cations.

Typical activation profiles, as seen for other mammalian muscles, were obtained for both the fast- and slow-twitch muscles from the young adult Tammar wallabies. The fast-twitch muscle fibres had a greatly different sensitivity to Ca^{2+} and Sr^{2+} (pCa_{50}-pSr_{50} = 1.32 ± 0.02 (mean ± SEM), n=5) whereas the slow-twitch muscle fibres had a similar sensitivity to these divalent cations (pCa_{50}-pSr_{50} = 0.47 ± 0.01, n=9). In addition, a small proportion of fibres from both fast- and slow-twitch muscles had combined activation characteristics i.e. these data could not be fitted with a single exponential curve suggesting that a mixture of fast- and slow-type regulatory isoforms coexist in these fibres.

During development the separation between the force-pCa and force-pSr curve of the fast-twitch muscle was significantly less than that observed in the adult muscle. This separation was 1.14 ± 0.03 (n=6) at 64 days and progressively increased to 1.24 ± 0.02 at 131 days. The fast-twitch muscle became progressively less sensitive to Ca^{2+} during development; pCa_{10} values decreased from 6.78 ± 0.06 (n=6) at 64 days to 6.36 ± 0.04 (n=7) at 131 days. These values were significantly higher than those obtained from fibres from the young adult (6.03 ± 0.02, n=4, P<0.05). The sensitivity of the contractile apparatus to Sr^{2+} also decreased during development i.e. pSr_{10} values decreased from 5.68 at 64 days to 5.09 at 131 days pouch life. In the muscles taken from pouch young at 64, 103 and 131 days, fibres with combined characteristics were not found.

This study provides one of the first accounts of the developmental profiles of muscles from a marsupial. During development of the fast-twitch muscles there appears to be changes in the regulatory protein complex of these fibres. In the adult animals there are fibres with mixed activation characteristics.

West, J.M., Barclay, C.J., Luff, A.R. & Walker, D.W. (1999) Journal of Muscle Research and Cell Motility, 20, 249-264.

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*Department of Zoology, The University of Melbourne, Australia.

This project was partly funded by the NH&MRC (Australia).

REGULATION OF THE CALCIUM RELEASE CHANNEL FROM SKELETAL MUSCLE BY THE NUCLEOTIDES ATP, AMP, IMP AND ADENOSINE

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Calcium release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs) determines skeletal muscle force. With severe metabolic fatigue, calcium release from the SR is impaired in conjunction with a depletion of phosphocreatine and a fall in cytosolic [ATP]. Under these conditions ADP is hydrolysed to AMP rather than being reconverted to ATP. The AMP is normally deaminated to inosine monophosphate (IMP) by myoadenlyate deaminase. Individuals with myoadenlyate deaminase deficiency (MDD) cannot convert AMP to IMP so that more AMP gets hydrolysed to adenosine. Adenosine has been shown to inhibit calcium release in permeablised and skinned muscle fibres whereas IMP does not significantly inhibit calcium release (Duke & Steele, 1998; Blazev & Lamb, 1999). The tendency of MDD individuals to accumulate myoplasmic adenosine is believed to contribute to their increased susceptibility to muscle fatigue (Blazev & Lamb, 1999). We investigated the regulation of single RyR channels by various nucleotides to determine their mechanism of action.

SR vesicles were prepared from the back and leg muscles of New Zealand rabbits killed by captive bolt prior to muscle removal. Vesicles were incorporated into planar lipid membranes separating two solutions: *(cytoplasmic)* 100 nmol/l $Ca^{2+}_{(free)}$ (1 mmol/l $CaCl_2$ plus 4.5 mmol/l BAPTA), 10 mmol/l TES (pH7.4), 230 mmol/l Cs methanesulfonate (CsMS), 20 mmol/l CsCl plus various concentrations of ATP, AMP, IMP and adenosine; *(luminal)* 1 mmol/l $CaCl_2$, 10 mmol/l TES (pH7.4), 30 mmol/l CsMS, 20 mmol/l CsCl.

In the absence of nucleotides RyRs were inactive at 100 nmol/l cytoplasmic Ca^{2+}. Addition of ATP strongly activated RyRs to an open probability (P_o) of 40% with half maximal activation occurring at a concentration (K_a) of 600 μmol/l. AMP activated RyRs to a lesser extent (maximum P_o = 0.08, K_a= 2 mmol/l), adenosine produced slight activation (P_o ~0.01) and IMP produced no activation. AMP and adenosine competitively inhibited ATP-activated RyRs indicating that these nucleotides act at a common site on the RyR protein. Adenosine inhibited with a Hill coefficient of one and with half-inhibiting [adenosine] (K_i) at various [ATP] of: (K_i at [ATP] mmol/l) 0.05 at 0.2, 0.09 at 0.5, 1.12 at 2, 7.0 at 4 and >20 at 8. Thus for [ATP] between 0.5 to 4 mmol/l the inhibiting potency of adenosine was reduced four fold by each doubling of [ATP]. These data indicate that inhibition occurs by the binding of a single adenosine molecule preventing the binding of two ATP molecules. Hence there exist at least two ATP binding sites for RyR activation. In contrast, IMP up to concentrations of 8 mmol/l produced no inhibitory effect on RyRs activated by 0.5 or 2 mmol/l ATP.

In conclusion, adenosine and AMP competitively inhibited ATP activated RyRs whereas IMP had no effect. These effects of adenosine and IMP are consistent with their effects on muscle contraction and can now be understood in terms of their direct action on the calcium release channels. During muscle fatigue the average [ATP] can fall to ~3 mmol/l but may fall to considerably lower levels near the RyRs in the triad junctions. At low [ATP] the 50 μmol/l adenosine that can occur in the myoplasm of sufferers of MDD should strongly inhibit calcium release and hence muscle force. Normal individuals produce IMP, which does not interfere with calcium release, as an alternative to AMP and adenosine, which do interfere with calcium release and so exacerbate muscle fatigue.

Blazev, R. & Lamb, G.D. (1999) Muscle and Nerve, 22, 1674-1683.
Duke, A.M. & Steele, D.S. (1998) Journal of Physiology, 513, 43-53.

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SPECIFIC FORCE OF THE RAT EXTRAOCULAR MUSCLES, LEVATOR AND SUPERIOR RECTUS, MEASURED *IN SITU*

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Extraocular muscles (EOM) are characterised by their faster rates of contraction and their higher resistance to fatigue relative to limb skeletal muscles. One of the most perplexing issues in muscle physiology is why EOM generate significantly lower specific forces [sP_o, force per muscle cross-sectional area (CSA), kN/m^2] than skeletal muscles. In previous studies the examination of EOM contractility was generally performed on isolated muscles, *in vitro*. It is possible that during the intricate dissections required for *in vitro* investigation that surgical trauma results in damage directly to the EOM and this contributes to the disparity in sP_o values between EOM and skeletal muscles. In this study, we have re-examined the issue of whether EOM produce lower sP_o than skeletal muscles. Specifically, we have investigated the force producing capacity of the levator palpebrae superioris (levator) and superior rectus muscles, from the rat, *in situ*. We compared the values for absolute force (P_o) and sP_o with those for muscles studied *in vitro*. We tested the null hypothesis, that the sP_o for EOM obtained *in situ* would not be different from that of limb skeletal muscles. A corollary to our primary hypothesis was that P_o and sP_o for EOM obtained *in situ* and *in vitro* would not be different.

For the evaluation of EOM function *in situ*, Sprague-Dawley rats (250-450 g) were anaesthetised deeply with sodium pentobarbitone of (60-80 mg kg^{-1}, i.p.) such that they did not respond to tactile stimuli throughout the procedures. During the intricate dissection procedures, nerve and blood supply to either the levator or the superior rectus muscle remained intact. The EOM were attached to a force transducer and the cranial nerves exposed for direct stimulation. After determination of optimal muscle length (L_o) and stimulation voltage, a full frequency-force relationship was established for each muscle. In separate experiments, the levator and superior rectus muscles were `excised for evaluation of isometric contractile function *in vitro*, using methods described in detail elsewhere (Lynch *et al.*, 2000). Animals were killed by cardiac excision whilst still anaesthetised.

Maximum P_o for the levator and superior rectus muscles was 177 ± 13 mN and 280 ± 10 mN, respectively. For the calculation of specific force, a number of rat levator and superior rectus muscles were partially digested in a 20% nitric acid-based solution in order to isolate individual muscle fibres. Muscle fibre lengths (L_f) were expressed as a percentage of overall muscle length, allowing a mean L_f to L_o ratio to be used in the estimation of muscle CSA. Mean L_f:L_o was determined to be 0.38 for the levator muscle and 0.45 for the superior rectus muscle. The sP_o for the rat levator and superior rectus muscles measured in situ, was 275 kN/m^2 and 280 kN/m^2, respectively. These values are within the range of sP_o values commonly reported for rat skeletal muscles. Furthermore, P_o and sP_o for the levator and superior rectus muscles measured *in situ* were significantly higher ($P < 0.001$) than P_o and sP_o for these muscles measured *in vitro*.

The results indicate that the force output of intact EOM differs greatly depending on the mode of testing. *In situ* evaluation yields higher forces such that sP_o values are similar to those for limb muscles. Most skeletal muscles develop similar forces *in situ* and *in vitro*, whereas EOM generate far less force in all studies performed *in vitro*. Although *in vitro* evaluation of EOM contractility will continue to reveal important information about this group of understudied muscles, the lower sP_o values of these preparations should be recognised as being significantly less than their true potential. We conclude that EOM are not intrinsically weaker than skeletal muscles.

Lynch, G.S., Hinkle, R.T. & Faulkner, J.A. (2000) Experimental Physiology 85, 294-298.

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Ca^{2+} DYNAMICS IN INTACT MAMMALIAN EXTRAOCULAR MUSCLES

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Extraocular muscles (EOMs) are a functionally diverse group of muscles responsible for both rapid (saccadic) and slow (pursuit and vergence) eye movements. The diversity of the EOM properties may result from the extreme demands placed on them by the wide dynamic range of the oculomotor system. This functional diversity of these muscles is reflected in their contractile protein composition with muscles containing up to six different myosin heavy chain (MyHC) isoforms (with a high incidence of individual muscle fibres coexpressing multiple MyHC isoforms). Despite being amongst the muscles with the fastest speed of contraction, they are also relatively resistant to fatigue. However, little is known of the Ca^{2+} release and uptake processes or the force-calcium relationship of EOMs. This is largely due to the fact that the large amounts of connective tissue within these complex muscles, and the accompanying difficulty for mechanical dissection, have almost completely precluded use of the vast array of muscle preparations employed in studies of muscle physiology. In particular, the inability to mechanically dissect viable intact (or even skinned) muscle preparations has prevented in-depth investigation of the Ca^{2+} release and uptake processes or the force-calcium relationship of EOMs. The aim of the present study was to circumvent these limitations by taking advantage of the spatial and temporal discrimination provided by a novel confocal spectrofluorimeter that was used for all fluorescence measurements.

Male and female Sprague-Dawley rats (230-520 g), were killed after deep anesthesia with sodium pentobarbitone (60-80 $mg.kg^{-1}$, i.p.). Each superior rectus (SR) muscle was removed intact, with the insertion preserved on a rectangle of sclera/cornea and the origin disinserted. Muscles were loaded in Carbogen-bubbled Kreb's Ringer for 30 min with 10 μM Fluo-5F/AM - a fluorescein-like Ca^{2+} sensor, with a dissociation constant of 2.57 μM in our experimental conditions. Muscles were rinsed and attached to an isometric force transducer (Research Grade 60-2999, Harvard Apparatus Inc, South Natick, MA, USA.), on the stage of a Nikon upright microscope. A Viewscan DVC-250 (BioRad Direct View) system attached to the microscope was the basis of the confocal spectrofluorimetry system. An argon ion laser 25 mW provided 488 nm excitation of a stationary slit aperture, which was scanned over the muscle specimen at approximately 25Hz. Nikon water immersion (dipping) lenses (10 or 40×) immersed close to the muscle in the bathing solution collected emitted fluo-5N fluorescence. Force-Ca^{2+} relationships were derived from force-frequency (1-150 Hz) data acquired from intact EOMs at optimal length.

The dynamics of Ca^{2+} dynamics evident in contracting EOMs were well resolved with spatial and temporal properties of the confocal recording system. Perhaps surprisingly, the rise and decay times of Ca^{2+} transients were not significantly faster than those of the fast-twitch extensor digitorum longus muscles from mice. However, Ca^{2+} levels in maximally stimulated EOMs commonly increased to 5-6 μM, significantly in excess of the levels apparent in isolated fibres or whole limb skeletal muscles. This clearly indicates higher absolute Ca^{2+}-release and sequestration rates in the EOM. In addition, force-Ca^{2+} curves were less steep that in limb muscles, a factor that may contribute to the ability of the EOMs to generate graded force responses.

Our observations of high levels of intracellular free [Ca^{2+}] during the contraction of EOM, coupled with previous observations of a high total calcium content (Porter & Karathanasis, 1999), extensive sarcoplasmic reticulum development and small fibre size may help explain the rapid development of force in the EOM. Study of the specialised functional roles of EOM continues to uncover molecular, developmental and structural specialisations that are dedicated to supporting functional specialisation.

Porter, J.D. & Karathanasis, P. (1999) Biochemical and Biophysical Research Communications, 257, 678-683.

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*W.K. Kellogg Eye Center, Department of Ophthalmology, The University of Michigan, Ann Arbor MI 48109-0714, USA.

^{1}H NMR STRUCTURE AND ACTIVITY OF A FORTY AMINO ACID PEPTIDE (AB) CORRESPONDING TO A PART OF THE II-III LOOP OF THE SKELETAL L-TYPE CALCIUM CHANNEL (DHPR)

Daniel Green, Suzy Pace, Marco Casarotto and Angela Dulhunty, The John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory, 0200, Australia.

Excitation contraction (EC) coupling is the process linking electrical activation of the sarcolemma to the release of calcium from the intracellular calcium store in the sarcoplasmic reticulum (SR) of striated muscle. EC coupling in skeletal muscle is thought to involve a physical interaction between the ryanodine receptor (RyR) (located in the SR) and the L-type calcium channel or dihydropyridine receptor (DHPR) which is located in transverse tubule invaginations of the sarcolemma. Previously, the twenty amino acid A peptide of the DHPR II-III loop has been shown to activate the Ca^{2+} release from the SR (El-Hayek *et al.*, 1995) and RyR channels (Dulhunty *et al.*, 1999). An essential sequence for the activating ability of peptide A is a cluster of five basic amino acids: as the ability of peptide A to activate the RyR is greatly reduced once this cluster is disrupted. Activation also requires that the basic amino acids are contained in a helical structure (Casarotto *et al.*, 2000b).

Peptide AB is a forty amino acid peptide which contains the twenty amino acids of peptide A plus the next twenty amino acids in the sequence of the skeletal DHPR II-III loop. We examined the ability of peptide AB to evoke Ca^{2+} release from SR vesicles that were partially loaded with Ca^{2+}, using spectrophotometric techniques with antipyralazo III as a calcium indicator. The three dimensional structure of the AB peptide in solution was solved by ^{1}H NMR.

Unlike peptide A, peptide AB was not able to evoke Ca^{2+} release from SR vesicles. Initial structural studies show that the peptide forms two helical portions each spanning approximately 12-15 amino acids. These helices are separated by a beta turn and appear to run parallel to each other. A comparison between the three dimensional structures of peptides A (Casarotto *et al.*, 2000a) and peptide AB suggest that the five basic residues found to be critical for peptide A activation of RyRs are buried between the helices and no longer exposed on an outer surface in peptide AB. The removal of the basic residues from the surface of the peptide accounts for the loss of function in peptide AB.

Casarotto, M.G, Gibson, F., Pace, S.M., Curtis, S.M., Mulcair, M. & Dulhunty, A.F. (2000a) Journal of Biological Chemistry, 275, 11631-11637.

Casarotto, M.G., Green, D., Pace, S.M., Curtis, S.M. & Dulhunty, A.F. (2000b) Journal of Biological Chemistry, (in press).

Dulhunty, A.F., Gallant, E.M., Laver, D.R., Pace, S.M. & Curtis, S.M. (1999) Biophysical Journal, 77, 189-203.

El-Hayek, R., Lokuta, A., Arevalo, C. & Valdivia, H. (1995) Journal of Biological Chemistry, 270, 28696-28704.

THE EFFECT OF BUFFERING ADP CONCENTRATION ON SARCOPLASMIC RETICULUM CALCIUM HANDLING PROPERTIES IN MECHANICALLY SKINNED FIBRES OF THE RAT

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Studying the effects of ADP within a muscle fibre has previously been difficult to achieve because the buffering and maintenance of [ADP] requires the removal or inhibition of the enzymatic reaction involving creatine kinase and myokinase. In an attempt to circumvent this problem numerous investigators have removed creatine phosphate from experimental solutions in order to inhibit these reactions. However the removal of creatine phosphate (CP) itself may cause alterations to maximum Ca^{2+}-activated tension (Fryer *et al.*, 1995) as well as affect the SR Ca^{2+} handling properties (Duke & Steele, 1999). Here, we have used the creatine kinase reaction ADP + CP $\Leftrightarrow$ ATP + Cr with a K_{app} of 200 (Chase & Kushmerick, 1995), together with endogenous creatine kinase activity and known concentrations of CP, ATP and Creatine to buffer the ADP concentration.

Male Long-Evans Hooded rats were killed by halothane overdose. Single fibres from the extensor digitorum longus (EDL) muscle of the rat were mechanically skinned under paraffin oil and attached to a force transducer. With direct access to the myoplasmic environment the fibres were then bathed in standard solutions containing (mmol/l) 90 HEPES (pH 7.10), 50 HDTA, 8 ATP, 10 CP, 1 Mg^{2+}, 36 Na^{+}, 126 K^{+}. The amount of creatine added to solutions was varied to buffer ADP to the required levels. The sarcoplasmic reticulum (SR) of the skinned fibres was loaded with Ca^{2+} in solutions containing 0.4 mmol/l EGTA (Ca^{2+} = 0.2 μmol/l) and the SR Ca^{2+} was released with 30 mmol/l caffeine in the presence of 1.0 mmol/l EGTA and low Mg^{2+} (0.05 mmol/l). The relative amount of SR Ca^{2+} was estimated from the relative area under caffeine-induced force responses.

Buffering the [ADP] to a physiological resting level of 10 μmol/l was achieved by adding 3 mmol/l creatine to the loading solution. Even at such a low level of ADP the maximal SR Ca^{2+} loading was reduced to about 50% of that of the controls where [ADP] was less than 1 μmol/l. Moreover, when the preparations were loaded in the presence of 12 mmol/l creatine and the ADP was buffered to 40 μmol/l, under our conditions the SR was only able to maximally load Ca^{2+} to 30% of controls. Interestingly, the removal of CP from solutions causes a similar depression in the ability of the SR to load Ca^{2+}, and it has been proposed that this effect was due to CP *per se* (Duke & Steele, 1999). However based on our results it is most likely that this apparent effect of CP is due to the increased presence of ADP because one could estimate that when CP is removed from solution the average [ADP] in the preparation during loading increases to about 40 μmol/l. The elevated ADP appears to reduce the SR Ca^{2+} loading ability by reducing the activity of the SR Ca^{2+} pump, and by dramatically increasing the leak of Ca^{2+} from the SR.

In conclusion, these results suggest that creatine can be used effectively to buffer ADP levels in the presence of a functional creatine kinase regenerating system, and that the reduction in SR Ca^{2+} handling ability of the SR is due to ADP accumulation rather than CP depletion.

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PROTEIN KINASE C-ε REGULATES CARDIAC Na^+-K^+ PUMP FUNCTION IN HYPERALDOSTERONEMIA

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Hyperaldosteronemia, often associated with cardiac hypertrophy and failure, induces a decrease in cardiac sarcolemmal Na^+-K^+ pump activity which may play a role in the pathogenesis of these conditions. Since pump activity is regulated by protein kinase C (PKC) and since aldosterone (Ald) can activate PKC we examined if Ald-induced PKC activation may explain the decrease in pump activity induced by hyperaldosteronemia.

Rabbits were anaesthetised with 2% Halothane and this was maintained during ventilation with a 2:1 mixture of N_2O and O_2. Osmotic minipumps containing either Aldosterone (50 μg kg^{-1} d^{-1}) or ethanol-vehicle (control) were implanted subcutaneously in the interscapular region. This dose of aldosterone produces a clinically relevant, ~3-fold increase in plasma Ald levels. After 7 days the rabbits were anaesthetised by intramuscular injection of xylazine 20 mg kg^{-1} and ketamine 50 mg kg^{-1} and a median sternotomy was performed to remove the heart. Ventricular myocytes were isolated, intracellularly dialysed and voltage-clamped with wide-tipped (~4-5 μm) patch-pipettes. Pipette filling solutions included 10 mM Na^+, a concentration near-physiological intracellular levels. After the whole-cell configuration had been established the myocytes were superfused with calcium-free Tyrode's solution, and K^+ channels were blocked by 2 mM Ba^{2+}. Na^+-K^+ pump current (I_p, normalized for membrane capicitance), arising from the 3:2 Na^+:K^+ exchange ratio was identified as the shift in holding current induced by pump blockade with 100 μM ouabain.

Mean I_p (±SE), normalised for cell capacitance and hence cell size, of 14 myocytes from 6 rabbits given Aldosterone (0.21 ± 0.02 pA/pF) was significantly lower than mean I_p of 6 myocytes from 4 vehicle-controls (0.32 ± 0.01 pA/pF). To achieve isoform-specific modulation of PKC we included in pipette solutions 100 nM peptide blockers to anchoring proteins for activated PKC. We selected the α- and ε-isoform peptide blockers since the α-isoform is reported to be translocated by Ald while ε-isoform is reported to regulate the sarcolemmal Na^+-K^+ pump. PKCε peptide inhibitor in the pipette solution abolished hyperaldosteronemia-induced pump inhibition (0.36 ± 0.02 pA/pF, *n*=12, 6 rabbits). The scrambled PKCε peptide inhibitor had no effect on Ald-induced pump inhibition (0.20 ± 0.01 pA/pF, *n*=9, 5 rabbits). To examine the role of PKCα in Ald-induced pump inhibition we included PKCα peptide inhibitor in pipette solutions. Mean I_p (0.21 ± 0.01 pA/pF, *n*=10, 5 rabbits) was similar to mean I_p measured using peptide-free pipette solutions.

We conclude that PKCε regulates cardiac Na^+-K^+ pump function in hyperaldosteronemia. This is likely to be of importance in hyperaldosteronemic states.

IDENTIFICATION OF G PROTEIN a-SUBUNITS MEDIATING M_3 AND P_{2U} SIGNAL TRANSDUCTION PATHWAYS USING REPLICATION-DEFICIENT ADENOVIRUSES

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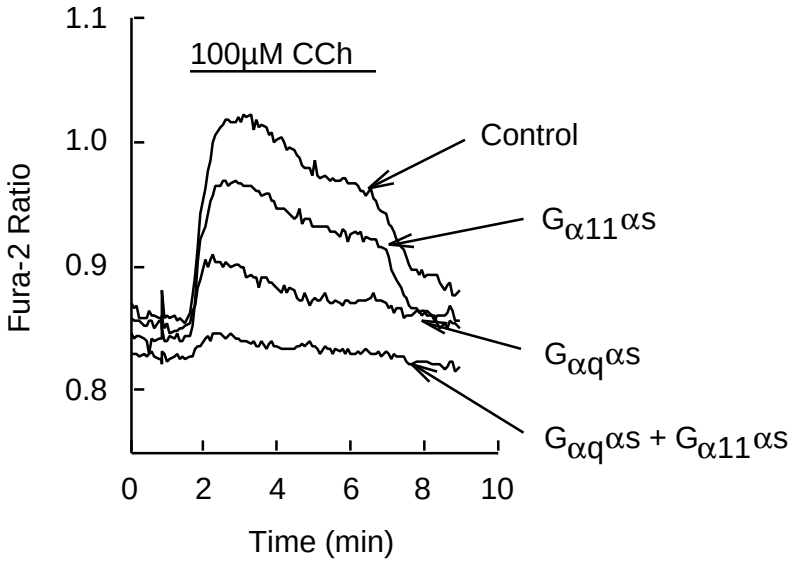
M_3 muscarinic receptors and P_{2U} purinergic receptors couple extracellular stimuli to increases in intracellular [Ca^{2+}] via phospholipase C-β and inositol 1,4,5-trisphosphate. The exact nature of these pathways, however, remains unknown, since the G protein α-subunits and phospholipase C-β isoforms that mediate them have not yet been identified. Our studies are aimed at identifying the G protein α-subunits involved in the muscarinic M_3 and purinergic P_{2U} signal transduction pathways using HT29 colonic epithelial cells as a model of colonic epithelia. We have used replication-deficient adenoviruses expressing wild-type or antisense G protein α-subunits to assess the involvement of specific G proteins in mediating increases in $[Ca^{2+}]_i$ in response to the activation of these receptors.

HT29 cells were infected with adenoviruses three days prior to fura-2 microspectrofluorimetry, which was used to quantify the $[Ca^{2+}]_i$ response to either the muscarinic agonist, carbachol (CCh) or the purinergic agonist, UTP. G protein α-subunit isoform specific antibodies were used with Western blotting and with immunochemistry using confocal microscopy to confirm the specificity of protein over-expression or suppression.

Previously, we have found that the M_3 response is mediated by G protein βγ-subunits, while the P_{2U} response is mediated by G protein α-subunits and that G_q has some involvement in mediating both the M_3 and P_{2U} signals (Cummins *et al.*, 2000). The results indicated, however, that G_q may be only partially responsible for mediating these responses, suggesting the involvement of other G protein/s.



We have now used an adenoviruses expressing wild-type or antisense $G_{\alpha 11}$ to evaluate the involvement of $G_{\alpha 11}$ in each pathway. Infection with an adenovirus expressing antisense to $G_{\alpha 11}$ reduced the $[Ca^{2+}]_i$ response following CCh addition, indicating a role for βγ-subunits released from $G_{\alpha 11}$ in mediating the M_3 response. Moreover, co-infection with antisense $G_{\alpha q}$ and $G_{\alpha 11}$ adenoviruses showed that the effect of the antisense $G_{\alpha 11}$ is additive to the effect for the M_3 response seen with the antisense $G_{\alpha q}$ adenovirus alone. These results lead to the conclusion that the βγ-subunits released from both $G_{\alpha q}$ and $G_{\alpha 11}$ mediate the M_3 signal.

Infection with the antisense $G_{\alpha 11}$ adenovirus caused the same degree of reduction of the P_{2U} Ca^{2+} signal as that achieved with the antisense $G_{\alpha q}$ adenovirus, and co-infection did not produce an additive effect, indicating that the decrease in the P_{2U} response seen with these viruses is due to a non-specific or promiscuous effect. Thus the P_{2U} pathway is mediated primarily by a G protein other than $G_{\alpha q}$ or $G_{\alpha 11}$. In future work we will assess the involvement of $G_{\alpha 14}$, $G_{\alpha 16}$, $G_{\alpha 12}$ and $G_{\alpha 13}$ in mediating M_3 and P_{2U} signal transduction pathways.

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EFFECTS OF THE INOSITOL TRIPHOSPHATE (IP_3) RECEPTOR ANTAGONIST 2-AMINOETHOXYDIPHENYL BORATE (2-APB) ON ARTERIOLAR MYOGENIC TONE AND REACTIVITY

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Arterioles exhibit a state of partial constriction or tone the extent of which is related to the intraluminal pressure. While this contractile response is known to depend on extracellular Ca^{2+} the contribution of intracellular sources is less certain. Knowledge of this component is essential to defining myogenic signal transduction pathways since release of Ca^{2+} from intracellular stores may be involved in multiple processes including initiation of contraction and modulation of ion channels. Studies of the involvement of sarcoplasmic reticulum Ca^{2+} stores in maintenance of arteriolar tone are made difficult by the fact that when placed in zero mM Ca^{2+} solutions cannulated arterioles rapidly loose intracellular Ca^{2+} and myogenic tone. This alters the mechanical state of the vessel and results in totally passive responses to subsequent changes in intraluminal pressure. Recent studies have reported that 2-APB is a small molecular weight, cell permeable, inhibitor of the IP_3 receptor thus providing a potential tool for examining the role of intracellular Ca^{2+} release in maintenance of spontaneous arteriolar tone.

Sprague-Dawley rats were anaesthetized with sodium thiopental (100 mg/kg b.w., intraperitoneal). Their cremaster muscles were removed and the animals were then killed. The muscles were placed in a cooled dissection chamber and a segment of the first order arteriole (passive diameter approximately 160 μm) microdissected from surrounding tissues. Isolated arteriole segments were cannulated on glass micropipettes, pressurized under zero flow and mechanical responses examined using video microscopy. Under these conditions arterioles typically exhibit spontaneous myogenic tone. For measurements of smooth muscle Ca^{2+}, vessels were loaded with the Ca^{2+}-sensitive dye fura 2-AM (1 μM) and fluorescence recorded using a photometer based system coupled to the microscope. Additional studies were conducted in endothelial cells (ECs), isolated from bovine aorta, cultured in DMEM (37^oC, 5% CO_2) and plated onto matrix-coated coverslips. Measurements of changes in EC Ca^{2+} were assessed using a video-based imaging system.

To assess effects of IP_3 receptor blockade on intracellular Ca^{2+} release in isolated cells, ECs were exposed to ATP (10^{-6}M) in the absence and presence of 2-APB (1 – 300 μM). In the absence of extracellular Ca^{2+} ATP induces a rapid and transient Ca^{2+} release from intracellular stores. 2-APB alone had no effect on EC basal intracellular Ca^{2+}. 2-APB dose-dependently inhibited the magnitude of the ATP-induced Ca^{2+} release peak; threshold dose approximately 10 μM and IC_{50} 30 μM. In intact arterioles (n = 9) 2-APB treatment resulted in a variable response with a tendency towards vasodilation. In arterioles (n = 4) from which the endothelial layer had been mechanically removed (verified by the lack of a dilator response to acetylcholine 10^{-5}M) 2-APB induced a concentration-dependent relaxation with an IC_{50} of approximately 100 μM. Despite causing dilation 2-APB (300 μM) did not cause total loss of myogenic tone. In apparent contrast 50μM 2-APB caused a significant shift to the right in the concentration-response curve for phenylephrine ($7.7 \pm 3.2 \times 10^{-8}$ vs $2.5 \pm 2.3 \times 10^{-5}$ M).

Collectively the data are consistent with 2-APB acting as an antagonist of IP_3-induced Ca^{2+} release from intracellular stores. 2-APB in intact arterioles appeared to produce opposing effects on the endothelium and smooth muscle resulting in inconsistent changes in diameter whereas in endothelial denuded arterioles it exhibited reproducible concentration-dependent vasodilation. As 2-APB appeared more effective in inhibiting agonist-induced responses compared to inhibition of myogenic tone it is concluded that the latter is less dependent on IP_3-mediated Ca^{2+} release mechanisms. Further, these data suggest that this compound will be a useful tool for studies of intracellular Ca^{2+} dynamics in isolated myogenic arterioles.

RELAXATION OF UTERINE CONTRACTIONS BY b-ADRENOCEPTOR ACTIVATION

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Agents that activate β-adrenoceptors are amongst the drugs of first choice in attempts to suppress uterine contractions during pre-term labour. Despite extensive study, the mechanisms by which relaxation is achieved remains unclear. There is lack of consensus regarding the involvement of the cAMP second messenger system, the occurrence or not of hyperpolarization and the nature of the ion channels involved, and the possible suppression of the sensitivity of the contractile apparatus to Ca^{2+}. We have addressed these issues by measuring membrane potential, tension, cytoplasmic Ca^{2+} ($[Ca^{2+}_i]$) and cAMP levels in uterine muscle obtained from pregnant ewes.

Uterine muscle was obtained from ewes undergoing surgery on days 70-141 of pregnancy (term, 145 days). Tissues were taken to the laboratory and strips (~ 5x0.5 mm) were prepared and mounted in an organ bath, in continuously flowing physiological saline solution, for either simultaneous measurement of membrane potential, using conventional intracellular microelectrodes, and isometric tension, or $[Ca^{2+}_i]$ and tension. Cytoplasmic Ca^{2+} was estimated in tissues loaded with fura-2 following incubation in fura-2 AM (5 μM) for 1 h. Larger strips (3-6 mg) were prepared for measurement of cAMP. Following stimulation, tissues were snap frozen in liquid nitrogen and stored for 2 weeks at –70°C. Cyclic AMP levels were measured using an enzyme immunoassay kit (Cayman). Salbutamol was used to stimulate β-adrenoceptors, forskolin (FSK) to activate adenylyl cyclase, PCO 400 to activate ATP-sensitive K^+ channels and glibenclamide to block these channels.

Salbutamol, FSK and PCO 400 all evoked concentration-dependent hyperpolarization which abolished spontaneously occurring action potentials and contractions. The hyperpolarizations were reduced to 16 ± 5% (*n*=8) by glibenclamide (10^{-6} M) and were completely abolished in the presence of glibenclamide plus ouabain (10^{-6} M). Blockers of large-conductance, Ca^{2+}-activated K^+ channels, iberiotoxin or charybdotoxin, were without effect on the hyperpolarizations. Salbutamol (just-maximal concentration, $3x10^{-7}$ M for 30 min) completely abolished the contractions evoked by oxytocin (OT) 10^{-11} M, suppressed for 7/30 ± 2 min the contractions induced by 10^{-10} M OT, and was completely ineffective against the contractions evoked by 10^{-9} M OT. In the presence of nifedipine, which abolished action potentials, the depolarization evoked by OT was clearly visible. Salbutamol or FSK failed to hyperpolarize the OT-induced depolarization. Pretreatment with salbutamol did not reduce the rise in $[Ca^{2+}_i]$ or the amplitude of the contraction evoked by 10^{-9} M OT (n=5). Raising K^+_o evoked an increase in $[Ca^{2+}_i]$ and contraction. Salbutamol was without effect on $[Ca^{2+}_i]$ but reduced the contraction to 57 ± 4% (n=7). Activation of cAMP-dependent protein kinase using Sp-cBIMPS (10^{-5} M), or suppression of cAMP degradation using caffeine or 3-isobutyl-1-methylxanthine (IBMX) also caused hyperpolarization and abolished spontaneous contractions. In tissues from 5 ewes, the levels of cAMP/mg protein were increased significantly from basal values of 2.5 ± 0.2 (SD) pmol to 5.6 ± 0.5 pmol by salbutamol and to 10.6 ± 1.1 pmol by FSK. IBMX in the presence of salbutamol or FSK markedly increased cAMP levels to 11.7 ± 0.7 and 13.0 ± 1.6 pmol, respectively.

In conclusion, stimulation of β-adrenoceptors or adenylyl cyclase or increasing cAMP resulted in substantial hyperpolarization of myometrium in pregnant sheep. This was associated with cessation of both action potentials and contractions. OT-induced contractions were considerably more resistant to suppression by these manoeuvres. The reason for this appears to be an inability to counter the conductance underlying the depolarization evoked by OT.

RENAL TUBULOINTERSTITIAL PATHOLOGY: EFFECTS OF HIGH GLUCOSE AND HYPOXIA

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Ischaemic injury is one of the major factors responsible for tubulointerstitial fibrosis in diabetic nephropathy. The complex paracrine interactions that occur in response to changes in glucose concentration and/or hypoxia between the different cell types that make up the tubulointerstitium of the human kidney remain poorly understood. In this study we investigate the independent and synergistic effects of exposure to high glucose and hypoxia on cultured tubulointerstitial cells from human kidneys.

Primary cultures of human proximal tubular cells (PTC) and cortical fibroblasts (CF) were studied either in isolation or in co-culture. Cells were exposed to media containing either 5 or 25 mM glucose for 72 hours prior to study. In the final 24 hours some cells were subjected to 2% O_2 for 8 hours and then incubated for a further 16 hours under standard conditions (20% O_2). Cells and media were collected and used for measurements of cell growth parameters, collagen synthesis, TGF-β1 production and expression of the matrix metalloproteinases MMP-2 and MMP-9. Results are standardised to the control values in cells cultured alone in 5 mM and 20% O_2.

Exposure to high glucose increased thymidine incorporation in PTC (137.9±12.1% (mean± S.E.M.); n = 8; P <0.05) with no significant effect observed in CF. Co-culture abolished the response in PTC (91.2±11.2 %; n = 7). Hypoxia alone reduced thymidine incorporation in both PTC and CF (70.0± 7.2%; n = 7; P <0.05 and 73.4 ± 6.7%; n = 7; P< 0.05). This suppression by hypoxia was maintained in both co-culture and high glucose. The protein content of PTC was not influenced by either co-culture, glucose concentration or oxygen tension. In CF, high glucose increased cell protein (120±5.25%; n = 9; P < 0.05) and this effect was maintained under hypoxia and co-culture. Collagen synthesis in CF was increased in high glucose (137.0±10.4%; n = 5; P <0.05). Co-culture in 5 mM glucose also increased collagen synthesis in CF (140.0±14.1%; n = 5; P<0.05), however, no independent or synergistic effects were observed in the presence of hypoxia. MMP-9 and MMP-2 secretion by CF was markedly suppressed in 25 mM glucose (76 ± 20% and 61±19%; n= 5) and under hypoxic conditions (80±10% and 50±14%; n= 5) respectively. Co-culture conditions caused pronounced increases above basal levels in both MMP-9 (900±75%; n = 7; P <0.0001) and MMP-2 (204± 54%; n=7; P<0.001). The increase in MMP production in co-culture was paralleled by an 4 to 5-fold increase in active TGF-β1 production under all co-culture conditions.

These results show that hyperglycaemic and hypoxic states differentially mediate PTC and CF growth parameters and that in the co-culture model of the tubulointerstitium, these parameters are altered by paracrine interactions. The increases in extracellular matrix production seen after exposure to high glucose or hypoxia is associated with alterations in MMP and collagen production by CF and TGF-β1 production by PTC.

PROLONGED EXPOSURE TO NORADRENALINE ACTIVATES TYROSINE PHOSPHORYLATION-DEPENDENT MECHANISMS WHICH IMPAIR ARTERIOLAR RELAXATION

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Contraction of arterioles to agonists and increased intravascular pressure has been shown to be dependent on the biochemical pathway involving Ca^{2+} -calmodulin induced myosin light chain phosphorylation. However, it is appreciated that other mechanisms such as Ca^{2+} sensitization and time-dependent phenomena including cytoskeletal reorganization may contribute to contractile responsiveness including to long periods of steady-state tone which are maintained with low energy requirements. With respect to the latter we hypothesized that if arteriolar smooth muscle exhibits time-dependent changes in cytoskeletal arrangement , this may be manifested as differences in rate of relaxation following short and long-term exposure to contractile agonists.

To test this hypothesis studies were conducted in cannulated arterioles isolated from rat cremaster muscle. Cremaster muscles were removed from anaesthetized (sodium thiopentone 100 mg/kg i.p.) Sprague-Dawley rats which were then killed. The muscles were placed in a cooled (4^{o}C) buffer-filled chamber for microdissection of arterioles. Arterioles were cannulated on micropipettes, pressurized to 70 mmHg, superfused with a physiological salt solution and studied by video microscopy. In an initial set of experiments (n=5) the rate of relaxation was measured following 5 min or 4 h exposure to 5 μM noradrenaline (NA). Maximal diameter was determined following superfusion with 0 mM Ca^{2+} buffer. The studies were then repeated in fura 2 loaded vessels to determine changes in smooth muscle intracellular Ca^{2+}. In separate experiments the effect of long-term NA exposure was determined after treatment of arterioles with either 1. the microtubule depolymerizing agent demecolcine (10 μM) (n=3 and 3 time controls) or 2. the tyrosine kinase inhibitor genistein (3 – 30 μM; n=5).

Arterioles constricted from 54.5±2.6% (% of maximum diameter) to 21.0±2.0% after 5 min NA and to 23.3±2.5% after 4 h NA. Washout after 5 mins exposure to NA dilated arterioles to 58.1±3.7% in 3.7±1.0 min compared to 32.1±3.2% at > 15 min after 4 h NA (P<0.001). Rapid relaxation (91.9±6.3%) of vessels exposed to NA for 4 hr was observed following superfusion with 0 mM Ca^{2+} buffer indicating that impaired relaxation was reversible and Ca^{2+} dependent. A similar enhancement of contraction was not observed when arterioles were contracted with 75 mM K^{+}. In both short and long-term NA exposure intracellular Ca^{2+} showed an initial peak increase followed by a decline towards baseline levels. Exposure to demecolcine alone caused significant vasoconstriction (P<0.01) and subsequently potentiated NA vasoconstriction, however, microtubule depolymerization did not prevent the effect of long-term NA exposure on relaxation. While genistein alone caused a concentration-dependent dilatation it did not affect the extent of the acute NA-induced contraction. In contrast during the 4 h exposure to NA genistein caused a time-dependent loss of tone such that at 30 μM genistein the contraction was lost after approximately 90 min.

The data indicate that prolonged exposure to a contractile agonist induces biochemical alterations which prolong time to relaxation following removal of the agonist. The potentiated contraction is Ca^{2+}-dependent, appears to involve tyrosine phosphorylation, but does not involve the polymerization state of the microtubule network.

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AUTOPHOSPHORYLATION OF SKELETAL MUSCLE RYANODINE RECEPTOR CALCIUM RELEASE CHANNELS IN LIPID BILAYERS: EFFECTS ON ACTIVATION BY A PEPTIDE FRAGMENT OF THE DHPR II-III LOOP

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The activity of ryanodine receptor (RyR) calcium release channels is modulated by many endogenous factors including Ca^{2+} and ATP, co-proteins such as FKBP12, co-valent modification by oxidation or nitrosylation, and by phosphorylation. ATP acts either as a ligand, or via phosphorylation. Phosphorylation by endogenous CaMKII inhibits amphibian RyRs without added calmodulin (Wang & Best, 1992) or mammalian RyRs following calmodulin addition (Hain *et al.*, 1994). Conversely, RyRs are activated when phophorylated by exogenous PKA or PKC (Hain *et al.*, 1994). An endogenous anchored PKA has been associated with cardiac RyRs (Marx *et al.*, 2000). We recently found that pig skeletal RyR channels are activated by a 20 amino acid peptide (**A**) (Thr^{671}-Leu^{690} of the II-III loop of the skeletal dihydropyridine receptor) in the absence of ATP, but not in its presence (Gallant *et al.*, 2000). The aim of the present study was to determine whether this effect on peptide **A**-induced activation was due to ATP-induced autophosphorylation of RyRs in bilayers and if it was, which endogenous kinase was involved.

Sarcoplasmic reticulum (SR) vesicles were prepared from skeletal muscle removed from rabbits previously killed by captive bolt, or from normal or malignant hyperthermia (MH) susceptible pigs, following anaesthetic overdose (Laver *et al.*, 1997). SR vesicle preparation and lipid bilayer techniques have been described (Laver *et al.*, 1997). RyR channel activity was recorded with 250/250 CsCl (mmol l^{-1} *cis/trans*), 10 mmol l^{-1} N-tris[hyroxymethyl]methyl-2-aminoethanesulfonic acid (pH 7.4 adjusted with CsOH) (*cis & trans*), 1 mmol l^{-1} $CaCl_2$ (*trans*) and 10^{-4} mmol l^{-1} Ca^{2+} in the *cis* solution.

Addition of 2 mmol l^{-1} ATP to the *cis* solution caused strong activation of normal and MH pig and rabbit RyRs (P_o increased from ~0.001 to ~0.5) which was not reversed by perfusion of ATP out of the *cis* chamber. Channel activity did not increase if ATP was added with 2 mmol l^{-1} *cis* Mg^{2+} (P_o remained at ~0.001), but increased (to P_o of ~0.5) when ATP and Mg^{2+} were perfused from of the *cis* chamber, and Mg^{2+}-inhibition was removed. The increase in activity with ATP was largely due to autophosphorylation of RyRs because activity was substantially reduced (to P_o of ~0.01) if acid phosphatase was added after ATP. In addition, use of the non-hydrolysable AMP-PCP or AMP-PNP (2 mmol l^{-1}) led to a much smaller increase in activity (from P_o = 0.001 to 0.01), which was reversed with AMP-PCP removal. The suppression of RyR activation by peptide **A** in the presence of ATP was also due to phosphorylation since the peptide activated RyRs if added in the presence ATP plus acid phosphatase.

This is the first report of activation of RyRs in bilayers after phosphorylation by an endogenous kinase. The results provide compelling evidence for a kinase associated with the native RyR and presumably tethered through a membrane anchoring protein. We are currently attempting to determine which kinase and anchoring proteins are associated with the RyR in the junctional face membrane.

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DIRECT MEASUREMENT OF SARCOPLASMIC RETICULUM CALCIUM DURING FATIGUE OF SINGLE MUSCLE FIBRES OF THE CANE TOAD

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Contraction of skeletal muscle depends on Ca^{2+} stored in the sarcoplasmic reticulum (SR) which is released by the action potential. In this study our aim was direct measurement of Ca^{2+} in the SR store ($[Ca^{2+}]_{SR}$).

We used the recently developed Ca^{2+} indicator fluo-5N in its acetoxy-methyl (AM) form. In this form the dye diffuses passively into the muscle cell. Upon the action of cellular esterases the Ca^{2+} sensitive and membrane impermeant form fluo-5N is liberated which is then trapped in various membrane-bound organelles such as the SR. Because the K_d of fluo-5N is 90 μM it is expected that most of its fluorescence originates in the SR where the Ca^{2+} concentration is ~ 1 mM. Adult cane toads (*Bufo marinus*) were killed by a blow to the head and double pithed. Single fibres from the lumbrical muscles were incubated in fluo5-N-AM for 1.5 hr at 35^{o}C and then washed at 35^{o}C for a further 1 hr. Muscle fatigue was produced by repeated short tetani continued until force had declined to 50 % (Kabbara & Allen, 1999).

Fluorescence imaging revealed that a substantial amount of the fluo-5N is located in the SR terminal cisternae. Upon depleting the SR Ca^{2+} store with caffeine (30 mM) and the SR Ca^{2+} pump inhibitor TBQ (20 *m*M), the fluo-5N signal fell dramatically, further indicating that most of the fluo-5N signal originates from the SR. During tetanic stimulation the SR Ca^{2+} signal declined rapidly and then recovered with a complex time course at the end of the tetanus. In the experiments below we estimated the resting SR Ca^{2+} and the decline of SR Ca^{2+} during a tetanus (Δ SR Ca^{2+}).

We measured SR Ca^{2+} at various times during fatigue and compared them with the 1st (control) tetanus. In the 10th tetanus the resting SR Ca^{2+} signal was significantly reduced by 11 ± 4% but Δ SR Ca^{2+} and tetanic tension did not change. In the middle of fatigue, the resting SR Ca^{2+}, the Δ SR Ca^{2+}, and tetanic tension were significantly reduced by 18 ± 8 % (*n*= 8), 22 ± 3 % (*n*= 11) and 19 ± 6 % (*n*= 6), respectively. At the end of fatigue, the resting SR Ca^{2+} and the Δ SR Ca^{2+} were significantly reduced by 47 ± 5% (*n*= 9) and 50 ± 2% (*n*= 11), respectively. The resting SR Ca^{2+}, the Δ SR Ca^{2+} and tension were all fully recovered after 5 min rest. Interestingly, the resting SR Ca^{2+} signal showed two phases of recovery: a fast phase, complete at 20 s, which amounted to 28 ± 6 % (*n*= 4) of the resting SR Ca^{2+} and a slow phase (19 ± 3 %; *n* = 4) which had returned to control by 5 min.

This study describes a new method for continuously measuring $[Ca^{2+}]_{SR}$ with good time resolution in intact single skeletal muscle fibres. We confirm earlier results using indirect methods (Kabbara & Allen, 1999) which showed that $[Ca^{2+}]_{SR}$ declines during fatigue and returns towards normal during recovery from fatigue. The slow phase of recovery of resting SR Ca^{2+} signal is consistent with the idea that Ca^{2+} precipitates with phosphates in the SR lumen during fatigue and then is released during recovery (Fryer et al, 1995).

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THE MECHANISM OF THE FAILURE OF CALCIUM RELEASE IN GLYCOGEN-DEPLETED CANE TOAD SKELETAL MUSCLE

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After prolonged exercise to exhaustion, there is a correlation between muscle weakness and reduced muscle glycogen concentration (Bergstrom *et al.*, 1967). Although this observation is well established, the underlying mechanism remains obscure. The most obvious possibility is that when glycogen is depleted, high energy metabolites such as ATP or PCr may be reduced or products of metabolism such as H^+ and P_i may accumulate and cause fatigue. However measurements of muscle metabolites have failed to support this hypothesis and suggested instead that Ca^{2+} release may fail for some reason in glycogen-depleted muscles (Vollestad *et al.*, 1988). We have investigated the mechanism of failure of Ca^{2+} release in cane toad muscles stimulated to fatigue in the absence of glucose.

Cane toads were stunned and killed by double pithing. Single fibres were dissected from the lumbrical muscles. Fatigue was produced by 500 ms tetani repeated at 4 s intervals in glucose-free Ringer. When force was reduced to 50 % of control, stimulation was stopped and the muscle allowed to recover for 20 min. These fatigue runs were repeated until the force declined to 50 % in less than 3 min. In some fibres intracellular Ca^{2+} was measured with indo-1 or intracellular Mg^{2+} with mag-indo-1. In some fibres intracellular glycogen was measured (Nguyen *et al.*, 1998).

We have previously shown that in the first fatigue run the decline of force is slow (8.1 ± 1.1 min) and that the reduced Ca^{2+} transients are accompanied by a reduction of the caffeine-releasable Ca^{2+} store (Kabbara & Allen, 1999). Conversely, in fibres subjected to repeated fatigue runs in glucose-free conditions until fatigue occurred in less than 3 min, the Ca^{2+} transients showed a similar reduction but the magnitude of the Ca^{2+} store was unchanged. In such fibres the average glycogen content was significantly reduced from control (176 ± 30 mmol glycosyl units/l) to 85 ± 9 mmol glycosyl units/l. There was no evidence of metabolic decline because (i) the $[Mg^{2+}]_i$ (which depends on MgATP) showed no significant change, (ii) the caffeine contracture (a measure of maximum Ca^{2+}-activated force which is sensitive to P_i) was larger after a final fatigue run compared to an initial fatigue run.

These experiments support the view that failure of Ca^{2+} release is an important mechanism in muscle fatigue associated with glycogen depletion. They further suggest that the mechanism of failure of Ca^{2+} release is not caused by global changes in metabolites or reduced Ca^{2+} stores. Instead we suggest either that glycogen has a 'structural' role and is required for normal Ca^{2+} release or spatially-localised changes in metabolites can occur and disrupt Ca^{2+} release.

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ARACHIDONIC ACID DECREASES DEPOLARISATION INDUCED FORCE IN MECHANICALLY SKINNED MUSCLE FIBRES FROM THE RAT

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Arachidonic acid is a fatty acid present in cell membranes and is released into the cell as the precursor for a range of biologically potent compounds, the eicosanoids. In this study we investigated whether arachidonic acid has an effect on excitation – contraction coupling in mammalian skeletal muscle.

Male rats (Long – Evans Hooded) were anaesthetised by halothane overdose and killed by asphyxiation. Single muscle fibres from the EDL were dissected and mechanically skinned under oil. The fibres were attached to a sensitive force transducer and placed in a K^+ - based solution mimicking the internal environment in a muscle fibre at rest, where the sealed T – system becomes normally polarized (Lamb & Stephenson, 1994). By transferring the fibre into a similar solution where all K^+ is replaced by Na^+, the T – system can be rapidly depolarised resulting in a force response (Lamb & Stephenson, 1994). By alternating solutions, fibres underwent a series of successive cycles of depolarisation and repolarisation. When arachidonic acid was present at 10 μmol/l force declined markedly. Within 2 – 6 minutes force was reduced to 50% and then declined to 0 within 2 further depolarisations.

Separate experiments in which Ca^{2+} was released from the sarcoplasmic reticulum (SR) indicate that 10 μmol/l arachidonic acid markedly decreased the SR Ca^{2+} content. This was supported by the observation that loading the SR with calcium resulted in recovery of arachidonic acid – induced decrease in force response. However, increased leak from the SR or a deficiency in SR Ca^{2+} loading was not sufficient to explain the effects of arachidonic acid on depolarisation force entirely suggesting that arachidonic acid may also have an effect on the T – tubule system.

There was a change in the effects of the stock of arachidonic acid stored at -30^oC in mercaptoethanol for more than one week, suggesting that not only arachidonic acid but also some of its degradation products also interfere with normal E – C coupling in skeletal muscle.

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THE EFFECT OF INCREASING LUMINAL CALCIUM ON SKELETAL MUSCLE CALSEQUESTRIN

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Depolarisation of the sarcolemma triggers Ca^{2+} release through the ryanodine receptor (RyR) calcium release channels in the sarcoplasmic reticulum (SR) of skeletal muscle. The protein responsible for calcium storage within SR is calsequestrin (CSQ), which is located wholly within the SR lumen. CSQ is tethered to the RyR by two anchoring proteins, triadin and junctin (Zhang *et al*, 1997).

We have reported that CSQ is an inhibitor of native RyR channels. Increasing luminal ionic strength resulted in an increase in RyR activity, probably due to dissociation of CSQ from the RyR complex, while the addition of purified CSQ to the luminal chamber reversed this activity increase (Beard *et al.*, 1999). To verify that this activity increase observed after raising luminal ionic strength was due to the dissociation of CSQ, we used an alternative method of dissociation, exposing CSQ to high concentration of luminal Ca^{2+}. Increasing luminal Ca^{2+} from the physiological [Ca^{2+}] of 1 mmol/l to ≥10 mmol/l has been shown to disrupt the polymeric formation and complex folding of CSQ, which can prevent it binding to the RyR (Zhang *et al*., 1997, Wang *et al.*, 1998). Thus removing CSQ from the RyR complex should eliminate the inhibitory action it has on the channel, thereby increasing channel activity. In addition, increasing luminal Ca^{2+} activates RyRs, by binding to calcium activation sites located on the RyR (Sitsapesan & Williams, 1995; Tripathy & Meissner, 1996). These two reports suggest that increasing luminal Ca^{2+} may alter RyR activity via two mechanisms.

Rabbit skeletal SR vesicles containing RyRs (isolated from back and leg muscle of New Zealand male rabbits killed by a captive bolt) were incorporated into artificial planar lipid bilayer membranes, which were formed across an aperture with a diameter of 150-200 μM in a delrin cup. The bilayer separates two chambers, *cis* (cytoplasmic) and *trans* (luminal). Solutions contained Ca^{2+} (1 mmol/l), $CsCl_2$ (20 mmol/l), cesium methane sulfonate (250/30 mmol/l; *cis/trans*) and TES (10 mmol/l).

RyR activity was measured as fractional mean current (I'_F), which is mean current (analysed over a 30 sec interval) normalised to the maximal single channel current. Increasing luminal Ca^{2+} from 1 mmol/l to 20 mmol/l, increased I'_F from 0.11 to 0.49, almost a 4.5-fold increase. Upon returning to 1 mmol/l Ca^{2+} (control conditions) via perfusion of the luminal chamber, I'_F reduced to 0.21. However, I'_F was still almost 2.3-fold higher than control levels. Additional experiments show that further addition of purified CSQ to the luminal chamber restored channel I'_F close to control levels.

These results suggest that the increase in channel activity did, in fact arise from two distinct mechanisms. One in which channel activity was increased by the activating affect of increased luminal Ca^{2+} and the second in which CSQ dissociated by elevated Ca^{2+}, further activating the channel.

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EFFECT OF L(+)-LACTATE ON VOLTAGE-SENSOR CONTROLLED Ca^{2+} RELEASE IN MECHANICALLY-SKINNED MUSCLE FIBRES

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High intracellular concentrations of lactate have been shown to inhibit Ca^{2+}- and caffeine-induced Ca^{2+} release from isolated sarcoplasmic reticulum (SR) and in mechanically-skinned fibres and to reduce the open probability of single ryanodine receptors in lipid bilayers (Favero *et al.*, 1997; Dutka & Lamb, 2000). We sort to clarify whether a high cytoplasmic lactate concentration inhibits voltage-sensor controlled Ca^{2+}release in functional fibres. Recently, high lactate concentrations have been shown to have a relatively small inhibitory effect (≤10% reduction in peak force) on depolarization-induced Ca^{2+} release in mechanically-skinned fibres elicited by ionic substitution (Na^{+}) of the bathing solution (Dutka & Lamb, 2000; Posterino & Fryer, 2000). Such depolarization-induced force responses are relatively slow (~500 ms time to peak) and produce near maximum force and may be insensitive to small reductions in Ca^{2+} release.

We have developed a technique whereby mechanically skinned fibres can be electrically stimulated to produce both twitch and tetanic force responses (Posterino *et al.*, 2000). By applying a transverse electrical field (50-60 V cm^{-1}) to the skinned fibre, action potentials are generated in the T-system and elicit Ca^{2+} release via the normal physiological pathway. Long-Evans hooded male rats were anesthetized with halothane (2% vol/vol) and killed by asphyxiation. Excised extensor digitorum longus (EDL) muscles were placed in paraffin oil. Single fibres were mechanically-skinned using microforceps and then mounted to a force transducer. The cytoplasmic environment could then be rapidly manipulated by changing bathing solutions. Fibres were placed in a standard K-HDTA solution (1 mM free Mg^{2+}; 8 mM total ATP at pH 7.1) and positioned between two platinum electrodes. By applying a single pulse (50 V/cm^{-1}, 2 ms), twitch responses were produced. Tetanic responses were produced by applying a 50 Hz stimulus.

30 mM lactate had no significant effect ($P > 0.05$) on either the rise time (rise time between 20% and 80% of peak force) or the peak height of tetanic responses (105 ± 5% and 100 ± 2% respectively, n=7) or twitch responses (104 ± 4% and 100 ± 2%, respectively, n=7). The peak size of the twitch responses (~60% maximum force) should be a sensitive indicator of Ca^{2+} release. The only significant change observed was a small (13%) slowing in the relaxation rate of twitch responses. This was most likely due to small differences in Ca^{2+}-buffering, as Ca^{2+}-uptake was not significantly altered in the presence of 30 mM lactate (114 ± 9%, n=8, $P > 0.05$).

These findings indicate that lactate *per se* does not directly contribute to the reduction in Ca^{2+} release observed in the later stages of muscle fatigue (Allen *et al.*, 1995), though we cannot rule out lactate accumulation having some indirect inhibitory effect (e.g. via osmotic swelling).

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IMPROVED FUNCTIONAL PROPERTIES OF REGENERATING SKELETAL MUSCLES FOLLOWING HYPERBARIC OXYGEN INHALATION

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While hyperbaric oxygen (HBO) inhalation is an accepted adjunctive treatment for several conditions characterised by reduced tissue oxygen tension, its effects on the regeneration of skeletal muscles following acute injury lack rigorous examination. We used intramuscular injections of bupivacaine to achieve full muscle degeneration, and subsequently examined the structural and functional properties of regenerating hindlimb muscles of control- and HBO-treated rats. At intervals post-injury, rats were anaesthetised deeply (sodium pentobarbitone 60-80 mg kg^{-1} *i.p.*) for the excision of experimental muscles, and later killed by cervical dislocation whilst still anaesthetised. The properties of excised muscles were tested as described previously (Gregorevic *et al.*, 2000).

At 14 days post injury, the regenerating fast-twitch extensor digtorum longus (EDL) and slow-twitch soleus muscles of untreated rats produced ~50% and ~65% lower values for maximum isometric force (P_o) compared with uninjured muscles, respectively. Additionally these regenerating EDL and soleus muscles were comprised of fibres ~30% and ~67% smaller in cross sectional area (CSA) than the fibres of contralateral uninjured muscles.

The regenerating EDL muscles of rats treated with 2 atmospheres absolute (ATA) HBO for 14 days showed no improvement in either structural or functional properties compared with injured muscles from untreated rats. In contrast, the injured EDL muscles of rats treated with 3 ATA HBO showed significant increases in peak specific force output (sP_o, HBO-treated 166.7 ± 2.8 vs. untreated 117.1 ± 4.9 kN m^{-2}) and regenerating fibre CSA compared with untreated, or 2 ATA HBO-treated rats. Regenerating soleus muscles from 3 ATA HBO-treated rats demonstrated increased peak force output compared with injured muscles from untreated rats (sP_o, HBO-treated 145.7 ± 6.6 vs. untreated 112.2 ± 9.3 kN m^{-2}), but not changes in regenerating fibre CSA. These novel findings demonstrate that periodic HBO inhalation modulates the structural and functional properties of regenerating skeletal muscles following non-ischaemic injury, in a dose- and fibre type-dependent manner.

In separate experiments we imposed an HBO-mediated oxidative stress on uninjured skeletal muscles to study adaptations resulting from modulation of the intracellular reduction-oxidation (redox) status. We found that daily treatment with 3 ATA HBO over 28 days increased the activity of the antioxidant metalloezyme Mn^{2+}-superoxide dismutase by ~241% in EDL, but not soleus muscles, compared with the muscles of untreated rats. We propose that this is a consequence of redox status shift following HBO administration.

Signal transduction mechanisms during cell proliferation and differentiation are known to be sensitive to redox modulation (Dalton *et al.*, 1999). We conclude that the enhanced physiological properties of regenerating skeletal muscles from rats treated with HBO are the manifestation of HBO-mediated redox modulation.

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EFFECT OF OVARIAN STEROID HORMONES ON CONTRACTION STIMULATED GLUCOSE UPTAKE IN RAT SKELETAL MUSCLE

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To examine the roles of 17-β-estradiol (E_2) and progesterone (Prog) in glucose metabolism, contraction-stimulated glucose uptake was studies in red (RQ) and white (WQ) quadricep muscle of female Sprague-Dawley rats. Groups included sham operated rats (C) and ovariectomized rats treated with placebo (O), E_2 (E), Prog (P), and both hormones at physiologic doses (P+E) or with a high dose of E_2 (P+HiE). Hormone (or vehicle only) delivery was via time release pellets inserted at the time of surgery, 15 days prior to metabolic testing. Rats were weight matched to 200 g and were given 20 g/day of rat chow so that there was no significant change in weight relative to C rats. On the morning of testing, fasted rats were given an intraperitoneal injection of 250 μCi of 2-[1-14]-deoxy-D-glucose (2DG) immediately prior to 30 minutes of treadmill exercise (0.35 m/sec), or rest. Rats were then killed by CO_2 suffocation and the tissues were removed and frozen in liquid nitrogen for later analysis. Plasma glucose and free fatty acid levels were similar between all treatment groups at rest and during exercise. Similarly, plasma insulin levels did not vary between the groups either at rest or during exercise. The Table shows resting and exercise values for 2DG uptake (10^{-5} dpm/min/100 g of tissue) in RQ and WQ.

	RQ		WQ	
	Rest	Exercise	Rest	Exercise
C	0.9 ± 0.05*	10.4 ± 0.54	0.6 ± 0.02*	2.2 ± 0.10
O	0.4 ± 0.03*	5.1 ± 0.42*	0.3 ± 0.02*	1.6 ± 0.06
E	0.8 ± 0.04*	12.7 ± 0.40	0.5 ± 0.02*	2.4 ± 0.14
P	0.4 ± 0.04*	4.5 ± 0.35*	0.2 ± 0.02*	1.4 ± 0.03 [a]
P+E	0.8 ± 0.06*	5.9 ± 0.17 [acf]	0.2 ± 0.03*	1.4 ± 0.16
P+HiE	0.9 ± 0.13*	14.0 ± 0.21 [bde]	0.6 ± 0.01*	2.2 ± 0.13

Means ± SE; $n = 5$ in each group

* Denotes main effect ($P<0.05$) exercise compared with rest

Superscript letters denote $P<0.05$ compared with:

[a] Intact Placebo (C) [b] Ovariectomized Placebo (O)

[c] Ovariectomized E_2 (E) [d] Ovariectomized Prog (P)

[e] Ovariectomized E_2 + Prog (P+E) [f] Ovariectomized E_2 (pharmacological) + Prog (P+HiE)

These results indicate that ovariectomy reduces contraction stimulated glucose uptake in red skeletal muscle, although a similar effect was not seen in white skeletal muscle. Treatment with E_2 restored glucose uptake to normal levels, while progesterone alone had no effect. Interestingly, when both hormones were administered in combination, progesterone negated the beneficial effect of E_2 at physiological concentrations of E_2, but not at pharmacological concentrations. These data suggest that the ovarian hormones play a significant role in glucose uptake, demonstrating that hormonal status (menarche, ammenhoria, menopause) needs to be considered in cases of altered glucose metabolism. Further research examining the mechanisms whereby the female sex steroids may affect glucose metabolism during exercise and in other stress conditions is warranted.

THE ROLE OF SK CHANNELS IN THE HYPOXIC RESPONSE OF FETAL ADRENAL CHROMAFFIN CELLS

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In vivo studies in the sheep have previously demonstrated that hypoxia acts directly on the immature adrenal to stimulate catecholamine secretion prior to the development of adrenal innervation (Cheung, 1990). This was thought to occur via closing of oxygen-sensitive potassium (K_{O_2}) channels leading to membrane depolarisation, Ca^{2+} entry through voltage-gated Ca^{2+} channels and subsequent catecholamine secretion. Studies on single cells *in vitro* have shown the presence of oxygen-dependent K^+ current in sheep chromaffin cells (Rychkov *et al.*, 1998), however the type of K^+ channel responsible for this is a major point of conjecture.

Pregnant ewes between 137 and 142 days gestation (term is 147 ± 3 days) were used for these experiments. Ewes were killed with intravenous pentobarbitone (8.1 g), the fetal adrenal gland was removed and the medulla was dissected free of the cortex. Cells were then separated using collagenase and deoxyribonuclease, plated on glass coverslips and maintained in culture.

Whole cell patch clamp revealed the presence of a Ca^{2+}-dependent K^+ current which was reduced by 36 ± 7% by hypoxia (P<0.05) at 40 mV. The reduction in current amplitude was not due to a diminished Ca^{2+} influx as voltage-activated Ca^{2+} currents did not alter during hypoxia. Apamin (200 nM), a specific blocker of small conductance potassium (SK) channels, reduced peak current at 40 mV by 36 ± 8% (P<0.01), not significantly different from the reduction caused by hypoxia. In the presence of apamin, the absolute reduction of the current amplitude by hypoxia was very much reduced, indicating the likely involvement of SK channels in the oxygen sensing mechanism. TEA (1 mM) reduced the Ca^{2+}-dependent K^+ current and decreased the current suppression due to hypoxia, indicating that BK channels in these cells are also involved in the oxygen-sensing mechanism.

Cytosolic [Ca^{2+}] was measured using Fluo-3 and confocal microscopy. Hypoxia caused an influx of Ca^{2+}, with an increase in cell fluorescence of 27 ± 4% above normoxic levels (P<0.05). Apamin also caused an increase in cell fluorescence (24 ± 2%; P<0.001) which signifies that SK channels are open at resting membrane potentials. The application of both apamin and hypoxia together caused an increase in fluorescence of 21 ± 4% (P<0.001), not significantly different from the influx seen with either apamin or hypoxia alone. No influx of Ca^{2+} ions resulted from the addition of TEA (10 mM), indicating that TEA-sensitive channels could not be responsible for the initial hypoxia-induced depolarisation.

The patch-clamp and fluorescence data together suggest that oxygen-sensitive SK channels are responsible for the initial depolarisation induced by hypoxia and that oxygen-sensitive BK channels activated by the initial depolarisation modulate the response.

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TEMPERATURE DEPENDENCE OF CLC-1 CHLORIDE CHANNEL GATING RELAXATIONS

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ClC-1 channel activity is characterised by two separate gating processes of the same voltage-dependence, that can be dissected by virtue of their time course. Over a fast time-scale (milliseconds) each of the channels twin-pores are individually gated by voltage-sensitive chloride binding, while a slower process (operating over a course of tens of milliseconds) inactivates both pores simultaneously. In contrast, 'slow' gating in the *Torpedo* homologue, ClC-0 has reciprocal voltage dependence to the 'fast'-gating mechanism. ClC-0 type slow, hyperpolarisation-activated gating is strongly dependent on temperature, with an activation enthalpy ($\Delta H^{\ddagger}$) of ~290 kJ mol^{-1}, corresponding to a Q_{10}~40 (Pusch, Ludewig & Jentsch, 1997).

The aim of the current experiments was to assess the nature of ClC-1 gating transitions using temperature as a probe. Currents carried by WT hClC-1 transiently expressed in HEK 293 cells were measured using the whole-cell configuration of the patch-clamp technique. Current relaxations remained the sum of two exponentials and a steady-state offset at all temperatures. Both fast and slow components of current deactivation became faster with increasing temperature. Fast gating had an apparent activation enthalpy of 84±3 kJ mol^{-1}, with a corresponding Q_{10} of 3. The temperature dependence of the slow gating transition was only modestly greater, with $\Delta H^{\ddagger}$ 99±5 kJ mol^{-1},and a Q_{10} of 4. Apparent open probability as a function of membrane potential ($P_o(V)$) was shifted by approximately +20 mV in the temperature range from 20 to 30°C. The temperature dependence of the ClC-1 fast and slow gating relaxations intimates that the transitions are associated with relatively simple changes in protein conformation. The slow-gating process that simultaneously inactivates both ClC-1 pores is only moderately temperature dependent, in distinct contrast to ClC-0 slow gating. Hyperpolarisation activated gating resembling the ClC-0 slow-gating transition can be induced in ClC-1 under conditions of low internal, and external pH (Rychkov *et al.*, 1996). Hyperpolarisation activated gating of ClC-1 channel activity was recorded at pH_I 6.2, and pH_o 5.5. The $P_o(V)$ of this gating process was fitted with a Boltzmann distribution with half-saturation voltage -31±1mV, yielding a gating valence of ~1.3. Activating current was single-exponential in nature at all temperatures, and had $\Delta H^{\ddagger}$ 140±20 kJ mol^{-1}, with a corresponding Q_{10} of approximately 9. Deactivation of the current at +60mV followed a double-exponential time course, with $\Delta H^{\ddagger}$ 101±2 kJ mol^{-1} and Q_{10} of 4 for the fast component, and $\Delta H^{\ddagger}$ 140±20 kJ mol^{-1} and Q_{10} approximately 9 for the slow component. The half-saturation voltage of $P_o(V)$ for this gating process was independent of temperature. Hyperpolarisation activated gating of ClC-1, induced by high internal and external proton concentration has a strong dependence on temperature indicating that the transition involves a complex conformational rearrangement within the protein. In this respect, although the temperature dependence of this gating process is comparitively shallow, the pH-dependent hyperpolarisation activated gate of ClC-1 appears to be the counterpart of slow gating in ClC-0 chloride channel.

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FUNCTIONAL CHARACTERISATION OF RAT P2X7-EGFP FUSION PROTEINS

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The cytolytic P2X7 purinoceptor is an ATP-gated cation channel that can undergo conversion to a non-selective pore permeable to large organic molecules such as $NMDG^+$ and ethidium bromide (Petrou *et al.*, 1997). This "pore-dilation" has important implications in a number of immune responses and may even be involved in the development of the stroke penumbra. The aim of the present study was to examine the functional consequences of N or C-terminal EGFP (Enhanced Green Fluorescent Protein) fusion on the rat P2X7 receptor (rP2X7R) as a prelude to future studies on the regulation of pore-dilation.

Fusion constructs were made using C_1 (EGFP-rP2X7R) and N_1 (rP2X7R-EGFP) EGFP fusion vectors (Clontech). Functional characterization of channel and pore formation was conducted using confocal imaging and electrophysiological techniques. HEK-293 and COS-7 cells were transfected and fusion proteins were visualized (BioRad MRC-1024). ATP or BzATP in the presence of ethidium bromide (25 μM) were applied and the emissions of EGFP and ethidium were viewed in separate detector channels to monitor ethidium uptake (indicative of pore formation) and cellular localization of the EGFP tagged rP2X7R. rP2X7R-GFP. cRNA encoding fusion and wild-type receptors was injected into *Xenopus* oocytes (oocytes were isolated from *Xenopus laevis* using AEESC approved procedures) and electrophysiological properties were compared.

Two days following transfection of rP2X7R-EGFP in HEK-293 and COS-7 cells clear evidence of membrane localization was seen. With EGFP-rP2X7R a similar distribution was found in HEK-293 but in COS-7 cells the fusion protein was distributed diffusely throughout the cytoplasm. The functional consequences of GFP fusion in mammalian cells were assessed by a standard dye-uptake assay for pore-formation. In HEK-293 cells transfected with either fusion construct, addition of ATP or BzATP resulted in ethidium uptake within 3-10 minutes. Uptake rates were significantly enhanced when temperatures were increased from 22^{o}C to 37^{o}C. The time course and extent of ethidium uptake in cells expressing fusion constructs was identical to that seen in cells expressing rP2X7R alone and no ethidium uptake was seen in control (non-transfected) cells. Thus, EGFP tagged rP2X7Rs appear to localize to the membrane and retain the pore forming phenotype. Electrophysiological studies revealed that the inward current elicited upon agonist addition of the rP2X7R-EGFP fusion protein did not differ to that of the rP2X7R receptor suggesting that channel function was not altered by fusion. In conclusion, the channel and pore properties of the rP2X7R and GFP fusion proteins are identical to rP2X7R, making these EGFP fusions a powerful tool for future analysis of pore formation.

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RELEASE OF MITOCHONDRIAL Ca^{2+} VIA THE PERMEABILITY TRANSITION ACTIVATES ENDOPLASMIC RETICULUM Ca^{2+} UPTAKE

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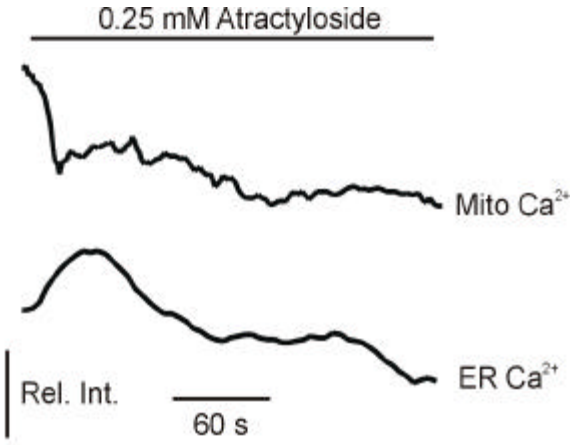
Communication between the endoplasmic reticulum (ER) and mitochondria is thought to be important in the regulation of intracellular free Ca^{2+} $[Ca^{2+}]_i$ and, amongst other functions, initiating cell death. We used new genetically expressed and targeted Ca^{2+} sensors (cameleons) to investigate the movement of Ca^{2+} between the ER and mitochondria of intact cells and focussed on the role of the mitochondrial permeability transition (MPT), in this interaction. We hypothesized that release of Ca^{2+} from mitochondria using an MPT agonist (atractyloside) would cause release of ER Ca^{2+}, perpetuating cellular Ca^{2+} overload and initiating cell death.

The cDNA for yellow cameleon 2.1 (YC2.1) and endoplasmic reticulum targeted cameleon-3ER provided by Roger Tsien (Howard Huges Medical Institute, University of San Diego). We constructed a mitochondrial targeted YC2.1 (YC2.1mito) by amplifying YC2.1 by the polymerase chain reaction (PCR) with primers encoding a mitochondrial targeting sequence (COX8). Human embryonic kidney 293 cells were transiently transfected with mitochondria and ER-targeted cameleons and were imaged with time resolved confocal microscopy.

Atractyloside-induced opening of the MPT resulted in specific loss of mitochondrial Ca^{2+} over a 4 min period that could be blocked by cyclosporin A (Figure). The ER initially (30 sec) sequestered this Ca^{2+} and later released it (60 sec post-MPT), leading to cellular Ca^{2+} overload (Figure). This cycle of ER sequestration and subsequent release could be totally abolished by blocking ER specific Ca^{2+}-ATPases using cyclopiazonic acid. We conclude that the ER plays an important role in moderating changes in intracellular Ca^{2+} following MPT and may play a key role in cell death initiated by mitochondrial mechanisms.



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THE EFFECTS OF VARIOUS MUTATIONS WHICH CONVERT RECOMBINANT GLYCINE RECEPTOR CHANNELS TO BEING CATION SELECTIVE

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Using the patch-clamp technique, we have already shown how three point mutations in the M2-pore region of the glycine receptor channel (GlyR) could convert the, normally anion-selective, glycine receptor channel to being cation selective (Keramidas *et al.*, 1999, 2000). This set of mutations, referred to as the 'Selectivity Triple Mutant (STM)', was the reverse set of mutations that was able to change the selectivity of the nicotinic acetylcholine channel (Galzi *et al.*, 1992). The triple mutations were of the cytoplasmic alanine 251 to a glutamate (A251E), the threonine 265 to a valine (T265V) and the deletion of the cytoplasmic proline 250 (P250Δ). In that study and in the present one, GlyRs were expressed in HEK293 cells and whole cell and single channel currents were examined using patch clamp techniques at 20 ^{o}C. The STM, which had extremely small glycine activated currents and no clearly discernible single channel currents, needed very high GlyR expression to get measurable responses, had a P_{Cl}/P_{Na} permeability ratio of 0.27 and no measurable calcium permeability. Following the above study, the responses to three other sets of GlyR mutations were also explored.

First, as we had predicted (Keramidas *et al.*, 2000), the T265V mutation in the STM was actually counter-productive for channel activation and a Selectivity Double Mutation (SDM) of the GlyR, including only the A251E and P250Δ mutations, was more sensitive to glycine, even more cation selective, had much larger whole cell currents than the STM and more clearly-defined single channel currents. The SDM was also somewhat permeable to Ca^{2+}, with P_{Ca}/P_{Na} = 0.34, and single channel currents were outwardly rectifying. The latter response was consistent with the positively charged extracellular ring of arginines (R271) still lining the pore and reducing the concentration of cations in the extracellular GlyR vestibule. When this positively charged R271 was then mutated to a neutral alanine (SDM+R271A), there was an increase in single channel conductance for inward current, eliminating rectification, and an increase in Ca^{2+} permeability (P_{Ca}/P_{Na} = 0.74). A subsequent mutation of the R271 to a glutamate to form a negative ring (SDM+R271E), continued to increase single channel conductance for inward current, converted the rectification to inward and further increased Ca^{2+} permeability (P_{Ca}/P_{Na} = 0.83).

We have therefore shown that the two mutations (T265V and P250Δ) are sufficient to convert the GlyR to cation selectivity and that mutating the extracellular positively charged ring to either a neutral or a negative one increased conductance for inward currents, as predicted from electrostatic considerations, and increased the calcium permeability of the channel.

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Supported by the National Health and Medical Research Council of Australia

DIVERGENT REGULATION OF INTERMEDIATE-CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS BY PROTEIN KINASES AND ATP HYDROLYSIS

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The smooth muscle intermediate-conductance K_{Ca} Channel (SMIK) is a recently cloned member of the IK1 gene family implicated in growth of vascular tissue during development and in pathological disease states (Neylon *et al.*, 1999). Analysis of the SMIK amino acid sequence reveals several potential mechanisms for modulation by ATP. First, multiple protein kinase consensus sites for protein kinase A (PKA), protein kinase G (PKG) and tyrosine kinases indicate that SMIK channels may be modulated by phosphorylation. Second, a sequence of 14 residues is found close to the C-terminus that is loosely reminiscent of a Walker A motif, the ATP binding site found in many ATP-regulated proteins. Thus, the aim of the present study was to investigate the role of ATP and protein kinase-induced phosphorylation in the regulation of SMIK channels. In these studies, wild type or mutant SMIK cRNA was injected into *Xenopus* oocytes and macroscopic currents on inside-out membrane patches recorded 4-10 days later.

In the presence of maximal concentrations of Ca^{2+} (10 μM), macroscopic currents ran down significantly (>60%) after patch excision but were restored upon inclusion of Mg/ATP (1 mM) in the bath solution. This enhancement of SMIK currents by ATP was Mg^{2+}-dependent and did not occur in the absence of Ca^{2+} or in uninjected oocytes. SMIK currents were not enhanced by non-hydrolysable analogs (AMP-PPP, AMP-PCP) or ATP-γS suggesting that the effect requires ATP hydrolysis. These results suggest that ATP regulates SMIK by direct interaction with an ATP hydrolysis site on the channel, however point mutation of the critical Walker A lysine residue (K400A) in SMIK did not attenuate the effect of ATP. Thus, the mechanism of ATP enhancement of SMIK currents is at present unknown.

Application of the catalytic subunit of PKA produced a 50-80% inhibition of current. This effect is Mg/ATP-dependent, but absent after boiling the kinase. In contrast, PKG produced a slight (20%) enhancement of SMIK currents. As several putative protein kinase consensus sites (PKA/PKG) are present in the proximal portion of the C-terminus, where calmodulin is thought to interact, it was hypothesized that protein kinases modulate SMIK by phosphorylating the calmodulin-binding domain (CBD). Thus, the putative CBD (54 amino acids) of SMIK was expressed as a GST fusion protein and the ability for it to be phosphorylated by PKA and PKG assessed in *in vitro* assays. [^{33}P]-phosphorylated bands were found in both PKA- and PKG-treated samples. Individual mutations of the three PKA/G sites did not abolish phosphorylation indicating that it can occur at multiple sites, however it was absent after mutation of all three consensus sites. We next examined whether mutation of all three sites in the full length channel had any effect on modulation by PKA. The degree of inhibition by PKA was identical for both triple mutant and wild-type channels. Thus, we have demonstrated that both PKA and PKG phosphorylate SMIK within the CBD, however this mechanism is unlikely to be responsible for the individual effects of these kinases on channel gating.

In conclusion, we have demonstrated that SMIK channels are regulated in divergent ways by protein kinases and ATP hydrolysis. Protein kinase signaling pathways may thus constitute a major mechanism of smooth muscle regulation by virtue of their effect on the gating properties of SMIK channels.

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Cl^--DEPENDENCE OF GATING OF MYOTONIC MUTANTS OF THE ClC-1 CHLORIDE CHANNEL

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Autosomal dominant myotonia congenita (Thomsen's disease) is caused by missense mutations in the gene encoding the major skeletal muscle Cl^- channel, ClC-1. These mutations shift the voltage dependence of ClC-1 gating to more positive potentials, and this accounts for the reduced chloride conductance (G_{Cl}) seen in myotonic muscle fibres. A remarkably similar myotonia can be produced with 2-(4-chlorophenoxy)propionic acid (CPP), the effect of which has been attributed to a decreased affinity of Cl^- for the ClC-1 gating site where it normally binds to enable channel opening (Aromataris *et al.*, 1999).

Considering the similarity in channel characteristics produced by CPP and dominant negative mutations, the aim of this study was to determine if these mutations affected the Cl^-dependence of gating of ClC-1. Two recently identified point mutations (F307S and A313T) (Kubisch *et al.*, 1998) which cause Thomsen's disease were introduced into hClC-1 by standard two-step PCR based site-directed mutagenesis, expressed in HEK293 cells and analysed by patch-clamping. The Cl^- and voltage dependent gating of these mutants was analysed and compared to wild type (WT) hClC-1. Voltage of half maximal activation ($V_{1/2}$) for WT in standard bath conditions of 178 mM Cl^- was -90 ± 0.7 mV. This was shifted by +74 mV to depolarising potentials in the F307S mutant and +113 mV in the A313T mutant. The P_o of WT and mutant ClC-1 was also dependent on the concentration of Cl^- in the bath solution with an approximately 60 mV shift per decade change in Cl^- concentration recorded for WT and both mutant channels. CPP produced a shift in P_o to depolarising potentials in both the WT and mutant channels. CPP applied externally at 3 mM concentration, shifted $V_{1/2}$ of WT channel by ~50 mV, but in F307S and A313T mutant channels the shift was much smaller. The K_d for CPP on WT hClC-1 was 1.3 ± 0.5 mM while in the mutant channels it increased to 4.6 ± 0.4 mM for F307S and 7.5 ± 1.2 mM for A313T. At any given value of $V_{1/2}$ in either the mutant channels or in WT in the presence of CPP more Cl^- was necessary in the bath solution to open the same number of channels in the membrane indicating a decrease in affinity for the gating site. Both the myotonic mutations and CPP predominantly affected the slow time constant of current deactivation and the relative amplitude of that component. This result is consistent with an effect of both on the slow 'common' gate of ClC-1.

The results of this study suggest that CPP and these dominant myotonic mutations in ClC-1 reduce G_{Cl} via a similar mechanism and are consistent with a decreased affinity of the ClC-1 gating site for Cl^-.

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AN ATP-SENSITIVE K^+ CONDUCTANCE IN INTRACARDIAC GANGLION NEURONES FROM ADULT RATS

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A change in neuronal activity when the ATP-sensitive K^+ (K_{ATP}) channel opener cromakalim was administered to adult canine intracardiac neurones *in situ* via their local blood supply suggests that intrinsic cardiac neurones express a K_{ATP} channel (Thompson *et al*., 1998). The K_{ATP} channel is regulated by intracellular ATP and serves to transduce changes in cell metabolism into changes in membrane potential.

We have identified a K_{ATP} conductance in isolated neurones from adult (≥6 week old) rat intracardiac ganglia. Adult rats were humanely killed by cervical dislocation and exsanguination. Membrane currents and voltage responses were recorded in dissociated neurones using the perforated-patch, whole cell recording configuration. In symmetrical (140 mM) K^+ solutions, externally applied levcromakalim (3 μM) activated an inward current at negative membrane potentials, in a concentration dependent manner, with an EC_{50} of 1.6 μM. The levcromakalim-induced current reversed close to 0 mV and was completely inhibited by 10 μM glibenclamide. Under current clamp conditions in physiological salt solutions, activation of K_{ATP} channels by levcromakalim caused a ~15 mV hyperpolarization from the resting membrane potential. Bath application of glibenclamide (10 μM) depolarized the neurone, indicating a K_{ATP} conductance is activated under resting conditions. In contrast, in intracardiac neurones isolated from neonatal rats, levcromakalim (10 μM) failed to activate a conductance change (n=7) and glibenclamide (10 μM) did not affect the resting membrane potential. The K_{ATP} conductance in adult rat intracardiac neurones was also activated under hypoxic conditions, whereby changing the perfusing solution to one which was bubbled with 100% N_2 for at least 2 hours previously, induced an inward current at negative membrane potentials which exhibited a similar I-V relationship to the levcromakalim-induced current. This hypoxia-induced current was blocked by 10 μM glibenclamide.

The activation of K_{ATP} channels in mammalian intracardiac neurones may contribute to changes in neural regulation of the heart and cardiac function during ischaemia-reperfusion.

Thompson, G.W., Horackova, M. & Armour, J.A. (1998) American Journal of Physiology, 275, H1434-1440.

THE RELATIONSHIP BETWEEN FRACTION OF CROSS-BRIDGES ATTACHED AND FORCE PER CROSS-BRIDGE IN ISOMETRIC CONTRACTION OF MOUSE FAST- AND SLOW-TWITCH MUSCLE

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The amount of force produced by myosin cross-bridges in muscle during contraction is a variable of fundamental importance to understanding mechanisms of contraction. In this study, the relationship between force per cross-bridge and the fraction of cross-bridges attached in an isometric contraction was estimated for fast and slow muscles of the mouse. The novel feature of this estimate was that it was based on data relating to the energetics of contraction during shortening.

Adult mice were rendered unconscious by inhalation of carbon dioxide and then killed by cervical dislocation. The soleus and EDL muscles were removed from the animal and small bundles of fibres were dissected from the muscles. Experiments were performed *in vitro* at 25°C. The energetic cost of contractile filament movement during shortening of fibre bundles was determined from measurements of power output and rate of heat production during isovelocity contraction. These data were used to estimate the amount of filament sliding generated for each ATP used by cross-bridges (Worthington & Elliott, 1996) at a range of shortening velocities. The rate of ATP use was estimated from measurements of rate of enthalpy output (power output + rate of heat output). The amount of filament sliding was calculated from shortening velocity.

For both muscles, filament displacements per ATP used increased with shortening velocity and were greatest at the highest shortening velocities used (~0.8 × maximum shortening velocity, V_{max}). The maximum values were ~1.3 nm for soleus and 0.9 nm for EDL. At a given velocity, either absolute or relative to V_{max}, more filament movement was generated for each ATP used in soleus muscle than EDL. These results can be accounted for by a mechanism in which more movement is generated by each cross-bridge cycle in soleus and/or more cross-bridges are attached in EDL than soleus at a particular velocity.

The relationships between force and velocity and between filament displacement per ATP and velocity were used to estimate the force produced per actin filament and to determine the possible combinations of cross-bridge force output and fraction of cross-bridges attached during isometric contraction. The possible relationships between force per cross-bridge and fraction of attached cross-bridges were similar for the two muscle types. If the force per cross-bridge is about 4 pN, as suggested by several types of experiment (Huxley, 2000), then 20 to 25 % of cross-bridges would be attached in an isometric contraction, with the figure being ~5 % greater in soleus than EDL.

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EFFECT OF TWO DIFFERENT PORTIONS OF THE II-III LOOP OF THE DIHYDROPYRIDINE RECEPTOR ON SINGLE RYANODINE RECEPTORS

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Mutagenesis experiments have shown that the 18 amino acid (aa) sequence ^{725}Phe-^{742}Pro of the II-III loop of the α-subunit of the dihydropyridine receptor is essential for skeletal muscle type excitation-contraction-coupling (ECC) (Nakai *et al.*, 1998). Saiki *et al.* (1999) have shown that the 37 aa sequence ^{724}Glu-^{760}Pro of the DHPR II-III loop (peptide C), which contains the essential segment, has no effect by itself on ryanodine receptor (RyR) activity as determined by measurements of Ca^{2+}-release, [^{3}H]ryanodine binding, and methyl coumarin acetamide (MCA) fluorescence. However, it was capable of reversing the increase in activity of the RyR by the 20 aa II-III loop segment ^{671}Thr-^{690}Leu (peptide A). We investigated the modulation of single RyR channels by peptide C alone as well as in combination with peptide A to determine possible mechanisms by which the interplay of these two peptides with the channel protein can occur.

Sarcoplasmic reticulum vesicles containing skeletal RyR of rabbits (killed by captive bolt prior to muscle removal) were incorporated into an artificial planar lipid membrane, with solutions (*cis*/*trans*): 100 μmol/l $CaCl_2$, 10 mmol/l TES, 230 mmol/l caesium methanesulfonate (CsMS), 20 mmol/l CsCl. Channel activity was recorded at –40 mV.

Exposure of the RyR to 5 μmol/l peptide A leads to activation of the RyR as reported previously (Dulhunty *et al.*, 1999). Peptide C alone had a biphasic effect on RyR activity. At a concentration of about 15 μmol/l it produced ~4-fold activation. At higher μmol/l concentrations a distinctive inhibition occurred characterised by few openings to only low conductance levels. The ratio of channels that inhibited upon exposure to peptide C was concentration dependent. Removal of peptide C from the bath solution and/or exposure of inhibited RyRs to activators (peptide A, ATP, Caffeine) led to a slight increase in channel activity, but in only about 50% of the experiments did channel activity return to >10% of the control current. Peptide C, in 7 out of 10 channels, was able to inhibit RyRs that had been activated by peptide A. The kinetics of the inhibition indicate that peptide C inhibited these channels by a mechanism that was not merely a reversal of the peptide A activation process.

In this single channel study we have discovered new mechanisms by which peptide C interacts with the RyR. The apparently disparate findings by Sakai *et al.* (1999) and us can be explained by these mechanisms. Simultaneous activation and inhibition of different RyRs by peptide C could result in no overall effect of peptide C on Ca^{2+} release and [3H]ryanodine binding. Peptide A might prevent only the activation of the RyR by peptide C (yet to be investigated) and not the inhibition by peptide C. If so, "reversal" of the activation by peptide A would be better described as a suppression of activation by peptide C. These mechanisms might also occur during ECC.

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CREATINE TRANSPORTER PROTEIN CONTENT, LOCALIZATION AND GENE EXPRESSION IN RAT SKELETAL MUSCLE

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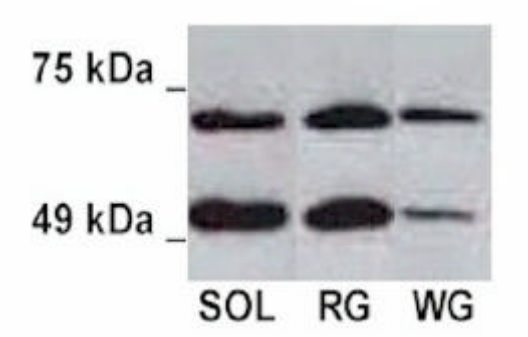
The present study examined the gene expression and cellular localization of the creatine transporter (CreaT) protein in rat skeletal muscle. Tissue was collected from previously killed male Wistar rats (n=6). Soleus (SOL), red (RG) and white gastrocnemius (WG) were analyzed for CreaT mRNA (Real Time RT-PCR), CreaT protein (Western blot) and total creatine (TCr) content (enzymatically). Cellular location of the CreaT protein was visualized using immunohistochemical analysis of muscle cross-sections. TCr was higher (P≤0.05) in WG than both RG and SOL, and RG was higher than SOL. Total CreaT protein content was greater (P≤0.05) in SOL and RG compared with WG. Two bands (55 and 70 kDa) of the CreaT protein were found in all muscle types as seen in the Figure.



Both the 55 kDa (CreaT-55) and the 70 kDa (CreaT-70) bands were present in greater (P≤0.05) amounts in SOL and RG compared with WG. SOL and RG had a greater amount (P≤0.05) of CreaT-55 than CreaT-70. Immunohistochemistry revealed that the CreaT was mainly associated with the plasma membrane in muscle types investigated, although some internal fluorescence was evident. CreaT mRNA expression per μg of total RNA was similar across the three muscle types. As in previous studies, we have identified a difference in the TCr content in different fiber types in rat skeletal muscle. In the present study we have shown that there may be an enhanced potential to transport Cr across the sarcolemma in the predominantly slow twitch (SOL) compared with fast twitch (WG) fibers. A recent study (Opt'Eindje *et al.* 1999) reported that, following Cr loading in rats, TCr content was increased in SOL but not altered in WG muscle. Furthermore, using incubated rat muscle strips, Willott *et al.* (1999) demonstrated that, at normal extracellular Cr concentrations, (e.g. 100 μM) SOL displayed a greater rate of Cr uptake than the extensor digitorum longus muscle. Interestingly, at high extracellular Cr levels (1 mM) the rate of Cr uptake was similar between both muscles. These data support the concept that an elevation in intracellular Cr results in a decreased expression of the CreaT protein. These data indicate that rat SOL and RG have an enhanced potential to transport Cr compared with WG despite a higher TCr in the latter.

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FIBRE TYPE PROFILE AND FUNCTIONAL ASPECTS OF THE RETRACTOR MAMMAE MUSCULATURE OF THE FEMALE MARSUPIAL *SMINTHOPSIS DOUGLASI* PRIOR TO AND DURING LACTATION

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The retractor mammae muscle is a specialised muscle terminating in the mammary tissue of female marsupials. It is subjected to altered functional demands during development of pouch young, particularly in pseudo-pouched marsupials, and is therefore expected to undergo changes to its fibre type profile over the time course of lactation. We have used the largest of the pseudo-pouched marsupials, the dasyurid *Sminthopsis douglasi*, to examine fibre type profiles (using Ca^{2+}/Sr^{2+}-activation characteristics and myosin heavy chain (MHC) typing) of retractor mammae muscles at three critical time points during lactation: 36-39 days (very small young permanently attached to teat), 51-53 days (mass of young greatest while still attached to teat) and 68-71 days (weaning age).

Animals were killed by suffocation following exposure to halothane in accordance with La Trobe University animal ethics procedures. Retractor mammae muscles were carefully removed, blotted on filter paper and placed under paraffin oil. Skinned fibre preparations were mounted between a force transducer and a pair of jewellers forceps and activated using experimental solutions containing different ionized concentrations of Ca^{2+} ($7.2<pCa<4.7$) or Sr^{2+} ($6.4<pSr<3.5$) as described previously (Fink *et al.*, 1986). Solutions had a free Mg^{2+} concentration of 1 mmol/1 and were matched for all other ionic species as well as for ionic strength. All experiments were performed at 22±1 °C. Following the contractile experiments, single fibres were saved and their MHC isoforms separated on SDS-PAGE using the protocol of Bortolotto *et al.* (2000).

Contractile results showed the presence of a large number of hybrid fibres, which was substantiated using MHC typing. In the non-suckled state, 62% (18/29) of fibres were classified according to Ca^{2+} and Sr^{2+} activation properties as $hybrid_{fast}$, i.e. fibres exhibiting both fast and slow activation properties, but mainly fast. Remarkably, we were unable to identify any $hybrid_{fast}$ fibres in 36-39-day suckled animals, but there was a concomitant increase in the number of fast-type (fast activation properties only) fibres (7/9 fibres) as well as an increase in $hybrid_{slow}$ (primarily slow with some fast activation properties) fibres (2/9 fibres). For 51-53-day suckled animals, the shift in fibre profile was away from the fast-type (3/7 fibres) and $hybrid_{slow}$ (0/7 fibres) fibres to include $hybrid_{fast}$ (1/7 fibres) and, more significantly, an increase in slow-type (slow activation properties only) fibres (3/7 fibres). Finally, in 68-71-day suckled animals, there were fewer slow-type fibres (4/18 fibres) and $hybrid_{fast}$ fibres (1/18 fibres) and a much higher percentage of fast-type fibres (13/18 fibres).

The fast-to-slow-to-fast cycle of adaptation of the muscle fibres seen here is indicative of the ability of the retractor mammae musculature to undergo changes in its fibre type profile in response to altered weight bearing demands as pouch young increase in size.

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FUNCTIONAL PROPERTIES OF PERMEABILIZED FIBRES FROM THE SKELETAL MUSCLES OF AGED RATS

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Aging is associated with a progressive loss of motor function, a slowing of muscle movements, and a decline in muscle strength. These age-related changes in muscle properties contribute to the increased incidence of fall-related injuries in the elderly, that often resulting in a loss of functional independence. Although some studies have investigated these age-related effects at the single muscle fibre (cellular) level (Thompson & Brown, 1999; Frontera *et al.*, 2000) and reported decreases in the maximum force producing capacity (P_o) and maximum velocity of shortening (V_{max}), other potential contributing factors have not been evaluated critically. These include a possible age-related decrease in the number of participating crossbridges during contraction as well as changes in the function of the sarcoplasmic reticulum (SR) of isolated fibres (Wang *et al.*, 2000). In this study we investigated the Ca^{2+}-activated contractile characteristics of fast muscle fibres from young and old rats. The purpose was to examine the effect of aging on: 1) Ca^{2+}-sensitivity, determined from the force-pCa relationship; and 2) the rigor force response of isolated fibres, to estimate the number of crossbridges participating in the maximum force response.

All experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne. Young (4 months of age, $n = 6$) and old (25 months of age, $n = 6$) male rats were anaesthetised deeply with sodium pentobarbitone (60 mg kg^{-1}, i.p) and the extensor digitorum longus (EDL, fast-twitch) muscle surgically excised. The rats were then killed. Membrane permeabilized fibres were dissected from muscles that had been stored in a glycerol-based chemical skinning solution (-20°C) for up to 6 weeks. Maximum Ca^{2+}-activated force (P_o), Ca^{2+}-sensitivity, and rigor force were determined using well-described techniques (Lynch *et al.*, 1995).

No differences were observed in P_o, specific P_o (kN m^{-2}), Ca^{2+} sensitivity, or rigor force of permeabilized fibres from fast-twitch muscles of young compared with old rats. The steepness of the force-pCa relationship (indicative of the level of cooperative interactions within the thin filaments) was decreased in fibres from old compared with young rats ($P < 0.05$). The results indicate that the underlying mechanisms responsible for the deficit in maximum P_o of intact skeletal muscles of old compared with young rats, are not associated with the force producing capacity of the contractile apparatus, but likely to be attributed to age-related impairments in excitation-contraction coupling, possibly the release of Ca^{2+} from the SR. Further studies comparing the SR Ca^{2+} release response of mechanically skinned fibres from the muscles of young and old rats will test this hypothesis.

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MEMBRANE CHOLESTEROL MANIPULATION AFFECTS EXCITATION-CONTRACTION COUPLING IN TOAD SKELETAL MUSCLE

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Cholesterol is unevenly distributed between the plasma and internal membranes of eukaryotic cells, including muscle cells. Cholesterol is known to affect the physical properties of cell membranes and also the function of several ion channels, pumps and receptors (Yeagle, 1985; Bastiaanse *et al.*, 1997). However, it is not known whether membrane cholesterol content affects the normal excitation-contraction (E-C) coupling process in skeletal muscle. Therefore, we investigated the importance of membrane cholesterol content to E-C coupling in toad skeletal fibres with the cyclic oligosaccharide, methyl-β-cyclodextrin (MβCD), which is widely used to manipulate membrane cholesterol content without causing damage to the lipid bilayer.

Cane toads were stunned with a blow to the head and then doubled pithed. Either intact bundles of fibres or mechanically skinned fibres were prepared from the iliofibularis muscle. The preparations were either attached to a force transducer or were loaded with fluorescent dyes and examined under the confocal microscope. The intact fibre bundles were bathed in a Na^+-based solution and stimulated electrically. Skinned fibres were bathed in a high [K^+] solution and subsequently the sealed t-system was depolarized in a Na^+-based solution. To study the Ca^{2+} handling properties of the sarcoplasmic reticulum (SR), the SR was loaded with Ca^{2+} in the presence of 0.5 mmol/l CaEGTA/EGTA and released with 30 mmol/l caffeine in the presence of 0.5 mmol/l EGTA. The area under the caffeine-induced force response was used as an indicator of Ca^{2+} content.

In skinned fibres, depletion of membrane cholesterol with MβCD caused a dose- and time-dependent decrease in t-system depolarization-induced force responses (TSDIFR). TSDIFRs were completely abolished within 2 min in the presence of 10 mM MβCD but were not affected after 2 min in the presence of a 10 mM MβCD: 1 mM cholesterol complex. There was a very steep dependence between the change in TSDIFRs and the MβCD: cholesterol ratio at 10 mM $M\beta CD_{total}$, indicating that the inhibitory effect of MβCD was due to membrane cholesterol depletion and not to a pharmacological effect of the agent. Tetanic responses in bundles of intact fibres were abolished after 3-4 h in the presence of 10 mM MβCD. The duration of TSDIFRs increased markedly within 2 min after the application of 10 mM MβCD and 10 mM MβCD: cholesterol complexes. The Ca^{2+} handling abilities of the SR were also modified after 10 min exposure to 10 mM MβCD and the Ca^{2+}-activation properties of the contractile apparatus were minimally affected by 10 mM MβCD. Confocal imaging revealed that the integrity of the t-system was not compromised by either intra- or extracellular application of 10 mM MβCD and that a high [Ca^{2+}] gradient was maintained across the t-system. Membrane cholesterol depletion also caused rapid depolarization of the polarized t-system as shown independently by spontaneous TSDIFRs induced by MβCD and by changes in fluorescence intensity of an anionic potentiometric dye in the presence of MβCD. This rapid depolarization of the t-system by cholesterol depletion was not prevented by blocking of the Na^+-channels with TTX (10 μM) and the L-type Ca^{2+}-channels with Co^{2+} (5 mM).

The results demonstrate that cholesterol is important for maintaining the functional integrity of the t-system and SR, probably by having specific effects on different membrane proteins that may be directly or indirectly involved in E-C coupling.

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MYOSIN HEAVY CHAIN ISOFORM EXPRESSION AND Ca^{+2}-STIMULATED ATPase ACTIVITY IN SINGLE SKELETAL MUSCLE FIBRES OF THE TOAD *Bufo marinus*

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Electrophoretically typed fibres from *Rectus abdominis* (RA) muscle of the cane toad *Bufo marinus* were used to test the hypothesis that in amphibian skeletal muscle there is a close relationship between Ca^{2+}-stimulated ATPase activity and MHC isoform composition. This hypothesis was formulated on the basis of histochemical data obtained by Rowlerson and Spurway (1988) with single skeletal muscle fibres from *Rana temporaria* and *Xenopus laevis.*

Eight adult (bw. 230 - 343 g) and 4 juvenile (bw. 11-14 g) toads were double pithed after 1h exposure to $4^{o}C$ to induce a comatose state, in accordance with procedures approved by the AEEC at Victoria University. Single fibres were dissected under oil, as described in Nguyen *et al.* (1998), from pelvic sections of RA muscles. Electrophoretic analysis of MHC isoform composition was carried out on all fibres (214) examined in this study using the Alanine-SDS-PAGE method of Nguyen and Stephenson (1999). According to Nguyen and Stephenson (1999), toad skeletal muscles contain four MHC isoforms: three twitch (BmHC1, BmHC2 and BmHC3) and one slow/tonic (BmHCT). A number of fibres (146) were divided in two segments, one was used for MHC analysis and the other for Ca^{2+}-stimulated ATPase measurements. Ca^{2+}-stimulated ATPase activities were determined in skinned fibres at pCa ~ 4.8, using the method of Chifflet *et al.* (1988) with slight modifications.

Based on MHC isoform composition, the fibre population examined in the present work comprised 4 types of pure fibres (**T**, **t1**, **t2** and **t3**), expressing one MHC isoform only (BmHCT, BmHC1, BmHC2 and BmHC3), 4 types of 2MHC-hybrid fibres (**T + t3**, **T + t1**, **t1 + t2**, **t2 + t3**) co-expressing 2 MHC isoforms and 3 types of 3MHC-hybrid fibres (**T + t1 + t3**, **T + t3 + t2**, **t1 + t2 + t3**), co-expressing 3 MHC isoforms. The hybrid fibres represented the largest proportion (65%) of the total number of fibres examined. The value (mean ± SE) of Ca^{2+}-stimulated ATPase (mmol Pi/L fibre/min) in pure fibres from RA muscles of the cane toad decreased in the order: **t1** (64.30 ± 8.08), **t2** (49.40 ± 1.98), **t3** (28.14 ± 1.42) and **T** (8.72 ± 2.24), suggesting that fibre types **t1**, **t2**, **t3** and **T**, correspond to fibre types F1, F2, F3 and T5 reported by Rowlerson and Spurway (1988). The study of Rowlerson and Spurway (1988) contains no information on Ca^{2+}-stimulated ATPase activities in fibres expressing more than one MHC isoforms. In our study, MHC isoform composition and ATPase activity were found to be related in fibres co-expressing tonic and twitch MHC isoforms, but not in fibres co-expressing twitch MHC isoforms only.

The results of this study strongly suggest that (i) the majority of fibres in RA muscle of the cane toad express more than one MHC isoform and (ii) in RA muscle of the cane toad, the relationship between MHC isoform expression and Ca^{2+}-stimulated ATPase depends on the MHC isoform complement expressed by the fibre.

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INTERHEMISPHERIC DISSOCIATION OF MOTOR AND SENSORY CORTICAL ORGANISATION IN HEMIPLEGIC CEREBRAL PALSY

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There have been a number of physiological studies of motor recovery in hemiplegic cerebral palsy, which have identified the presence of novel ipsilateral projections from the undamaged hemisphere to the affected hand. However little is known regarding the afferent projection to sensory cortex and its relationship to the reorganized cortical motor output. We used transcranial magnetic stimulation (TMS) to investigate the corticomotor projection to the affected and unaffected hands in a group of 7 subjects with hemiplegic cerebral palsy, and also performed functional magnetic resonance imaging (fMRI) studies of the patterns of activation in cortical motor and sensory areas following active and passive movement of the hands.

Both TMS and fMRI demonstrated a normal contralateral motor and sensory projection between the unaffected hand and the cerebral hemisphere. However, in the case of the affected hand, the TMS results indicated either a purely ipsilateral projection, or a bilateral projection in which the ipsilateral pathway had the lower motor threshold, whereas passive movement resulted in fMRI activation in the contralateral hemisphere.

These results demonstrate that there is a significant fast-conducting corticomotor projection to the affected hand from the ipsilateral hemisphere in this group of subjects, but that the predominant afferent projection from the hand is still directed to the affected contralateral hemisphere, resulting in an interhemispheric dissociation between afferent kinesthetic inputs and efferent corticomotor output. The findings indicate that there can be differences in the organization of sensory and motor pathways in cerebral palsy, and suggest that some of the residual motor dysfunction experienced by these subjects could be due to an impairment of sensorimotor integration at cortical level as a result of reorganization in the motor system.

DEVELOPMENT AND EVALUATION OF AN ALFENTANIL-MORPHINE MIXTURE TO OPTIMISE POSTOPERATIVE ANALGESIA

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A common strategy for the treatment of acute post-operative pain is to use intermittent intravenous boluses of an opioid, such as morphine, administered at fixed intervals and titrated to analgesic effect. Dose regimens, however, are often empirically determined and restricted by the kinetic limitations of an individual opioid (Upton *et al.*, 1997). Pharmacokinetic modelling, targeting brain concentrations, has previously been shown to be effective in devising optimal dose regimens for anaesthetic agents acting in the CNS (Ludbrook & Upton, 1997). The aim of this study was to use similar techniques to devise an optimal opioid dose regimen for the treatment of acute post-operative pain, and to test its efficacy in the clinical setting.

The ideal characteristics of an opioid were considered to be rapid onset and slow offset of analgesia. As no opioid in clinical use seemed suitable, kinetic analysis of a combination of two opioids, alfentanil and morphine, was performed. It was assumed that the apparent CNS equilibrium half-times for alfentanil and morphine were 1 and 34 minutes, respectively, and that 10 mg of morphine and 0.75 mg of alfentanil were equipotent. Simulations, using a compartmental kinetic model and the "Scientist" program, were used to determine the effect on the profile of CNS concentrations of varying opioid proportions and dosing intervals. Kinetic analysis suggested a mixture of alfentanil:morphine in the proportions 7.5:0.5 mg, administered at 2 minute intervals, would allow achievement of target brain concentrations approximately 3 times faster than with conventional morphine pain protocols, but with a similar duration of effect in both cases. The theoretical optimal dose regimen was then compared to a standard morphine pain protocol. Patients undergoing elective surgery were randomised to receive either technique in recovery post-operatively. Pain was assessed using pain scores. The times until the patient reported being "comfortable", and until discharge from recovery, were recorded.

57 patients were studied, with 4 excluded because of interruptions in drug administration. One Way ANOVA revealed the groups were well matched for age, weight, sex and initial pain scores (P=0.52, 0.08, 0.94 and 0.86, respectively). Predictors of time to comfort were determined using general MANCOVA and the Visual General Linear Modeling module of the "Statistica" program. The total dose of drug (P<0.001) and the type of drug used (P =0.013), but not age (P =0.064), weight (P =0.91), initial pain score (P =0.25) or sex (P =0.45), were significant predictors of the time until the patient reported being "comfortable". The time taken to reach this point was 25 (SD 16) minutes for the mixture and 38 (SD 12.8) minutes for morphine, with a trend towards shorter times until discharge from recovery if the mixture was used (102 vs 125 minutes).

The use of kinetic analysis targeting the CNS allowed the development of a "virtual" drug, with a profile of effects not possible with any existing single agent. Use of this dose regimen can reduce the time a patient spends in pain post-operatively, and it has the potential to allow earlier discharge from the operating suite.

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CORTICOMOTOR REORGANISATION AND MOTOR RECOVERY AFTER STROKE

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As part of an investigation of the neurophysiological basis for motor recovery after stroke we performed transcranial magnetic stimulation studies of the corticomotor projection to the hand in 22 patients (29-72 yrs of age) with an anterior circulation stroke who recovered normal or near-normal motor function in the upper limb. Eleven patients were studied within 1 month of the stroke and 8 of these were then studied serially at 3 - 6 month intervals. Eleven patients who had suffered a stroke 6 months - 20 years previously were also studied. In addition to measurements of cortical excitability, long-latency intracortical inhibition and corticospinal conduction, the motor representation of the abductor pollicis brevis muscle was mapped on both the affected and unaffected side.

Cortical excitability and motor evoked potential (MEP) amplitude were commonly reduced on the affected side even after motor recovery in both the cortical and subcortical cases but particularly the latter. Evidence of cortical reorganisation, as shown by topographic shifts in the corticomotor maps, was frequently found in both patients with cortical and subcortical stroke who recovered motor function in the hand. The map shifts were present as early as 2 weeks in some patients with subcortical stroke. Map position returned progressively towards normal over the next 1-3 years in half the patients studied serially but was still abnormal in some patients studied 3-20 years after the stroke. Evidence of reorganisation on the unaffected side was also found in some patients with cortical stroke but not in subcortical cases.

The present findings indicate that cortical reorganisation commonly occurs both in patients with cortical and subcortical stroke who recover motor function. Whereas in patients with subcortical lesions reorganisation occurs only on the affected side, in cortical cases the changes may be bilateral. We postulate that cortical reorganisation is one of the mechanisms underlying the recovery of motor function after stroke and may compensate for persisting impairment of conduction in motor pathways in patients with subcortical lesions.

LOSING SPEED TO GAIN CONTROL? THE INFLUENCE OF MUSCLE RECEPTOR ORGANS ON TAILFLIPPING IN CRAYFISH (*CHERAX DESTRUCTOR*)

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Animal movement results from the interaction of internal motor pattern generators and external sensory influences. Invertebrates (e.g., crayfish) provide excellent opportunities to study this interaction at the level of small cellular level. In crayfish, muscle receptor organs (MROs) are identified proprioceptors that respond to the flexion of a single abdominal joint. It has been hypothesised that the MROs mediate local resistance reflexes (e.g., Fields, 1966), but they do not appear to do so in intact, freely behaving crayfish (McCarthy & Macmillan, 1999). The stretch-sensitive sensory neurons of the MROs run the entire length of the nerve cord (Bastiani & Mulloney, 1988; Macmillan & Vescovi, 1997), and some tailfan motor neurons receive direct input from MRO stretch receptors (Vescovi *et al.* 1997). These facts suggest that MROs are involved in coordinating whole body movements, particularly ones involving the tailfan. One such behaviour is tailflipping, which incorporates both repeated abdominal flexion and opening and closing of the tailfan.

Crayfish (*Cherax destructor*) were purchased from local suppliers. Animals were anaethestised by chilling on ice for 30 minutes prior to surgery to ablate the MROs or implant electromyogram (EMG) recording electrodes. External markers on the abdomen were used to locate the MROs. A small hole was drilled in the abdominal exoskeleton, and the nerve leading from the MROs to the abdominal nerve cord was cut. We ablated the MROs in abdominal segments 2-5 in this way; the remaining MROs in abdominal segments 1 and 6 could not be ablated without damaging the abdominal muscles. We recorded (EMGs) from the lateral abductor muscle of the exopodite (tailfan appendage). Crayfish were affixed to a post and induced to tailflip by gently squeezing the legs or tailfan with forceps. The EMGs were recorded digitally on computer, then rectified and smoothed prior to measurement.

MRO ablation significantly affected the period of tailflipping (ANOVA, $n = 18$, df = 2,15, $F = 3.83$, $P < 0.05$), as shown in the Table. Tailflipping was faster in individuals without MROs.

MROs	Mean period ± SD	*n*
Ablated	147 ± 26 ms	5
Intact, no surgery	249 ± 77 ms	8
Intact, sham surgery	233 ± 76 ms	5

The result suggests that crayfish use MROs to monitor and adjust abdominal position during tailflipping. Although tailflipping is most often used to escape from threat and is expected to be rapid, it appears that speed is sacrificed for increased control of the behaviour.

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LEVELS OF IMMUNOREACTIVITY FOR SYNAPTOSOMAL-ASSOCIATED PROTEIN OF 25kDa (SNAP-25) ARE HIGHER IN PERIVASCULAR AXONS OF AUTONOMIC VASODILATOR NEURONS THAN IN ADJACENT VASOCONSTRICTOR AXONS

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Soluble N-ethylmaleimide-sensitive fusion protein (NSF), NSF attachment proteins (SNAPs), and their receptors located on membranes of synaptic vesicles and nerve terminals (SNAREs), together constitute the basic molecular machinery for the exocytotic release of neurotransmitters (Söllner *et al.*, 1993). It is now known that botulinum toxins interfere with neurotransmitter release by cleaving specific SNARE proteins. Early studies showed that botulinum toxins interrupt neurotransmission from different classes of autonomic neurons to varying degrees (see MacKenzie *et al.*, 1982). Therefore, we have begun to examine the role of SNARE proteins in neurotransmission from functionally distinct classes of autonomic neurons by examining immunoreactivity (IR) for the target-SNARE, SNAP-25, in vasoconstrictor and vasodilator axons in two vascular beds.

Guinea-pigs (n=55) were killed by stunning and exsanguination before removal of the brain, lingual arteries or uterine arteries. Proteins were extracted in 0.1% sodium dodecyl sulphate prior to polyacrylamide gel electrophoresis (SDS-PAGE), and were transferred to nitrocellulose for demonstration of SNAP-25-IR using Sternberger SMI81 or Chemicon MAB331 monoclonal antibodies recognizing the N-terminus of SNAP-25, or Affinity Bioreagents PA1-740 rabbit antibody recognizing the C-terminus of SNAP-25. IR was detected using secondary antibodies conjugated to horse-radish peroxidase followed by enhanced chemiluminescence. Arteries were fixed and processed for cryostat sectioning and fluorescence immunohistochemistry. Transverse sections were triple-labelled for IR to tyrosine hydroxylase (TH), vasoactive intestinal peptide (VIP) and SNAP-25. Confocal microscopy was used to capture a single optical section of one field at the adventitia-medial junction containing both VIP-IR (vasodilator) and TH-IR (vasoconstrictor) varicose axons, from six sections per artery, from four animals. BioRad PIC files were imported into NIH Image as RGB stacks, and the levels of SNAP-25-IR (average optical density per pixel) in VIP-IR and TH-IR varicosities were recorded.

SDS-PAGE and immunoblotting with each SNAP-25 antibody revealed a single protein band at 25kDa in extracts of brain, lingual artery and uterine artery. The optical density of the SNAP-25-IR band was consistently greater in extracts of the lingual artery than in extracts of the uterine artery. Immunohistochemistry clearly demonstrated SNAP-25-IR in all VIP-IR varicosities in both arteries, but SNAP-25-IR was more variable in intensity in TH-IR varicosities. The mean level of SNAP-25-IR in VIP-IR varicosities in each artery was significantly higher than the mean level of SNAP-25-IR in adjacent TH-IR varicosities (Sternberger antibody, $F_{(1,523)}$ =172, P<0.0001; Chemicon antibody, $F_{(1,698)}$ =142, P<0.0001). Only a small proportion of TH-IR varicosities had high levels of SNAP-25-IR, particularly in the uterine artery. The mean intensity of SNAP-25-IR in TH-IR varicosities relative to adjacent VIP-IR varicosities was significantly lower in the uterine artery than in the lingual artery (Sternberger antibody, $F_{(1,523)}$ =12, P=0.001; Chemicon antibody, $F_{(1,698)}$ =18, P<0.0001).

These results demonstrate that IR for SNAP-25, a SNARE protein considered to be essential for calcium-dependent exocytotic release of neurotransmitters, is present at different levels in varicose axons of functionally distinct classes of autonomic neurons. Such variations in SNAP-25 levels may reflect a different probability of neurotransmitter release (see Barden *et al.*, 1999) from varicosities of different classes of perivascular axons, or a differential role of SNAREs in transmission from autonomic neurons utilizing different co-transmitters.

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SLOW SYNAPTIC POTENTIALS IN DESCENDING INTERNEURONS OF THE MYENTERIC PLEXUS IN GUINEA-PIG ILEUM STIMULATED BY DISTENSION

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Many interneurons and motor neurons in the myenteric plexus of guinea-pig ileum respond to distension of the intestine oral to their cell body with a burst of fast excitatory synaptic potentials (EPSPs) indicating that they lie in a descending reflex pathway. However, despite the widespread finding that electrical stimulation of internodal strands evokes slow EPSPs in many myenteric neurons, there has been little direct evidence that such synaptic responses can be seen as a result of physiological stimuli. Nevertheless, pharmacological studies of the descending reflex pathways suggest that tachykinins, which would be expected to cause slow EPSPs, may play a significant role in these pathways (Johnson *et al.*, 1998). This study was therefore aimed at determining if sEPSPs can be recorded in response to a distending stimulus, identifying the neurons that exhibit this response and determining whether tachykinins are involved.

Guinea-pigs were killed by stunning, followed by exsanguination. Segments of ileum were then removed, opened along the mesenteric border and partly dissected to expose the myenteric plexus at the anal end of a region in which the mucosa was intact. The preparation was placed in a partitioned organ bath enabling the cleared region to be superfused separately from the intact region. Distensions were applied to the intact region and recordings were made from neurons in the anal chamber. This allowed application of the antagonists to impaled cells, while leaving transmission in the rest of the reflex pathway unaffected. In addition, biocytin was added to the recording electrode to allow impaled cells to be characterized morphologically, and neurochemistry identified.

In 56 cells tested, 8 myenteric S-neurons gave a response characteristic of a slow EPSP. They had a depolarizing amplitude ranging from 5-10 mV, a rise time of 1-2 s, decay time of 15-60 s, no increase in cell conductance and increased excitability in that they evoked action potentials in some cells. In 7 of these neurons, a control slow EPSP could also be evoked by a 10 pulse 10 Hz train of electrical stimuli applied to a circumferentially directed internodal strand. The role of tachykinins in these slow EPSPs was tested by applying the NK_1 tachykinin receptor antagonist SR 140 333 (100 μM, 4 neurons) or the NK_3 tachykinin receptor antagonist SR 142 801 (100 μM, 2 neurons) to the recording chamber of the organ bath. Neither antagonist affected the slow EPSPs evoked by either distension or electrical stimulation. All 8 neurons that exhibited slow EPSPs in response to distension had descending axons and 6 of these neurons had side branches in other myenteric ganglia indicating that they were probably interneurons. Immunoreactivity for nitric oxide synthase was identified in 6 of the 8 neurons that had reflexly evoked slow EPSPs.

The results suggest that slow EPSPs are evoked by distension in a population of descending interneurons that are immunoreactive for nitric oxide synthase. However, these slow EPSPs are unlikely to be mediated by a tachykinin, raising the question as to the nature of the neurotransmitter responsible for this response.

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LOW-VELOCITY-EXCITATORY/HIGH-VELOCITY-SUPPRESSIVE CELLS IN CAT STRIATE CORTEX

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Substantial morphological and functional evidence indicates that area 17 (striate cortex, area V1) apart from the so-called X and W-inputs receives also direct input from the so-called Y relay cells in the dorsal lateral geniculate nucleus (see for review Orban, 1984). Despite this only a very small proportion of area 17 neurones responds to fast-moving visual stimuli (cf. Burke *et al.*, 1992). There is some functional evidence indicating that the apparent paucity of area 17 neurones responding to high-velocity visual stimulation is largely due to inhibitory interactions within area 17 (see for review Orban, 1984) and/or suppressive feedback from area 18 (Alonso *et al.*, 1993).

In the present study we have recorded single neurone activity from area 17 of eight anaesthetized (0.5 - 0.8% halothane in 67/33% of N_2O/O_2; EEG & ECG monitored continuously), paralyzed (gallamine triethiodide; i.v. 7.5 mg/kg/hr) and artificially respired adult cats. The concentration of halothane was adjusted to maintain slow-wave EEG record and keep the heart rate below 180 beats/min. In seven of these cats under ketamine (30 mg/kg, i.m)/Rompun (3 mg/kg, i.m) anaesthesia, we have made sharply circumscribed (~ 12^o in diameter) laser lesions of one retina. Single unit activity from area 17 was recorded 6 - 53 months after placement of the lesions. In every animal studied (including the cat without the retinal lesion) we recorded some cells which exhibited unusual velocity response profiles. These cells gave excitatory responses to optimally oriented elongated light bars moving across their receptive fields at velocities in the range of 2 - 20^o/s. Most of the cells responded poorly to the same stimuli moving at moderate velocities (40 - 100^o/s) while stimuli moving at high velocities (over 100^o/s) evoked purely suppressive, non-directionally selective responses. All the cells exhibited a substantial "spontaneous" activity (5 - 15 spikes/s) and had receptive fields at least 5^o from the *area centralis*. Furthermore, most of them, if not all, were located in the infragranular layer 5. In animals with retinal lesions all these cells were recorded from outside the lesion projection zone.

Recently, we have encountered cells with rather similar receptive field properties in the deep superficial layers and *stratum griseum intermediale* of cat superior colliculus (SC; Waleszczyk *et al.*, 1999). In view of the prominence of the X-input to area 17 we would argue that low-velocity-excitatory/high-velocity-suppressive (LVE/HVS) cells in area 17 receive their excitatory input predominantly from the X- information channel and their suppressive input from the Y-information channel. Furthermore, in view of their location predominantly, if not exclusively, in layer 5 where the cortico-tectal cells are located we would argue that LVE/HVS cells in area 17, like those in the SC, might play an important role in activation of "fixation/orientation" and "saccade" premotor cells in the SC.

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EFFECTS OF ACTIVATION OF DESCENDING PATHWAYS ON RESPONSES IN THE AUDITORY PERIPHERY

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The existence of a descending projection from the mammalian superior olivary complex (SOC) to the peripheral auditory receptor organ has been known for many years. The effects of activation of this "olivocochlear" system on the peripheral responses to sound has been studied primarily by electrical stimulation of the axons of the final output neurones at the floor of the IVth ventricle. More recently, anatomical tracing techniques have revealed that the olivocochlear neurones in the SOC receive direct descending input from higher centres, notably the auditory cortex and inferior colliculus. The effects of activation of these higher centres has not been systematically studied.

We chose to study the effects of electrical stimulation of the inferior colliculus on the peripheral responses to sound. Guinea pigs were anaesthetized with Hypnorm (fentanyl, 0.14 mg/kg; fluanisone, 4.5 mg/kg, i.m.) and Nembutal (pentobarbitone, 30 mg/kg i.p.) after a premedication with atropine (i.m.). Responses to brief tone bursts were monitored by a wire electrode placed near the round window of the cochlea. Electrical stimuli were delivered at varying depths in the inferior colliculus, using bipolar tungsten electrodes. Before electrical stimulation, animals were paralyzed by intramuscular injection of Pancuronium (0.2 mg). Heart rate was continuously monitored and supplementary doses of both Hypnorm (as above, every h) and Nembutal (15 mg/kg every 2 h) that were known to maintain deep surgical anesthesia in unparalyzed animals were routinely given. Heart rate never increased after paralysis, over the long term, nor during, or immediately after electrical stimulation. At the end of the experiments, animals were killed while still anaesthetized by intracardiac perfusion of fixative.

We found that electrical stimulation of the central nucleus and external cortex of the inferior colliculus produced changes in the neural and receptor potentials of the cochlea that were qualitatively indistinguishable from those elicited by stimulation of the olivocochlear axons at the floor of the IVth ventricle. Stimulation of the dorsal cortex of the inferior colliculus produced no effect. The effects of inferior colliculus stimulation were eliminated by surgical transection of the olivocochlear axons at the floor of the Ivth ventricle. We conclude that the descending pathways from inferior colliculus to the superior olivary complex produce sufficient activation of the olivocochlear neurones to affect the functioning of the peripheral receptor. Thus neural centres higher than the superior olive may participate in centrifugal control of auditory processing by affecting the earliest stages of transduction.

MICROINJECTION OF ANGIOTENSIN II INTO THE NUCLEUS OF THE SOLITARY TRACT ATTENUATES BAROREFLEX-MEDIATED CHANGES IN CARDIAC SYMPATHETIC NERVE ACTIVITY IN A WORKING HEART-BRAINSTEM PREPARATION OF THE RAT

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Angiotensin II (Ang II) acts in the nucleus of the solitary tract (NTS) to attenuate the reflex vagal bradycardia following baroreceptor stimulation (Paton & Kasparov, 1999). In the present study we determined whether Ang II in the NTS could also affect the cardiac sympathetic motor component of the baroreceptor reflex response.

An *in vitro* working heart-brainstem preparation (WHBP) was prepared from Wistar rats (80-120 g). Following deep halothane anaesthesia, animals were transected sub-diaphragmatically, decerebrated and perfused retrogradely, via the descending aorta, with Ringer's solution plus ficoll (1.25%) at 33^{o}C. Inferior cardiac sympathetic nerve activity (ICN) was monitored via a suction electrode. Activity was integrated and values expressed as percentages. Baroreceptor reflexes were stimulated by increasing perfusion pressure. Measurements of induced changes in heart rate and ICN activity were made from the linear portion of the input:output function curve. Ang II (500 fmol) was microinjected bilaterally into the NTS. Values quoted are mean ± SEM.

Basal perfusion pressure and heart rate were 72±5 mmHg and 316±18 bpm respectively (n=5). Phrenic nerve activity showed an incrementing discharge pattern indicative of adequate cerebral perfusion and oxygenation. The ICN exhibited ongoing activity that was modulated by inspiratory drive. Stimulation of the baroreceptors produced a marked depression of activity in the ICN by -41.8±4% from control (100%; n=5). Following microinjection of Ang II into the NTS there was an initial transient increase in ICN (15.0±5%). After this returned to baseline the baroreceptor reflex mediated inhibition of ICN was attenuated to –26.6±4% (P<0.05; Students t-test). The baroreceptor reflex inhibition of ICN activity recovered after a 10-15 min washout period. Losartan (an AT_1 receptor antagonist; 20 μM; n=5) prevented the Ang II induced attenuation of the baroreceptor reflex inhibition of ICN activity.

We conclude that Ang II at the level of the NTS attenuates the baroreceptor reflex-induced inhibition of sympathetic activity destined for the heart.

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THE RELATIONSHIP BETWEEN FLYING EXPERIENCE AND CARDIOVASCULAR PERFORMANCE DURING +Gz STRESS IN FIGHTER PILOTS

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Fighter pilots frequently report that their ability to tolerate applied G loads increases with regular exposure. Our previous work (Newman *et al.*, 1998; 2000) has shown that the cardiovascular system of +Gz-adapted fighter pilots responds differently to an orthostatic challenge than non-pilots. This paper presents the results of an investigation into the relationship between jet flying hours (as a marker of +Gz exposure) and the arterial blood pressure response to head-up tilt (as a marker of cardiovascular performance during +Gz stress).

14 male fighter pilots from RAAF base Williamtown participated in this study. Nine pilots had more than 1000 hours jet flying experience and 5 pilots had less than 500 hours jet flying experience. All pilots rested quietly in a supine position and then underwent a rapid (4 s) head-up tilt to +75 degrees. Arterial pressure was measured noninvasively on a beat-to-beat basis using a Finapres (Ohmeda) Blood Pressure Monitor during 2 minutes of supine rest and for 30 seconds in the upright position. For each subject, the change in MAP from mean resting values was obtained for the first 30 heart beats of the head-up tilt period. The average of the MAP tilt values were plotted against their flying hours, and the MAP responses across the 30 s of tilt were compared between the experienced and less experienced pilots.

There was a strong correlation (r=0.87, P<0.01) between the MAP response to tilt and jet flying hours. Comparison of the experienced (>1000 hours) and less experienced (<500 hours) pilots' responses to tilt indicates that the experienced pilots increased MAP more during the initial active tilt and maintained MAP at a higher level throughout the 30 s tilt. There were no significant differences between the two groups in terms of age, height or weight.

The results of this study support the anecdotal experience of fighter pilots and suggest that high performance flying experience is an important factor in determining the cardiovascular response to acceleration stress. These findings are important in the search for better ways of protecting pilots from the hazards of high +Gz flight.

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DIFFERENT PATTERNS OF PRE- AND POST-GLOMERULAR RESISTANCE RESPONSES TO REFLEX SYMPATHETIC ACTIVATION

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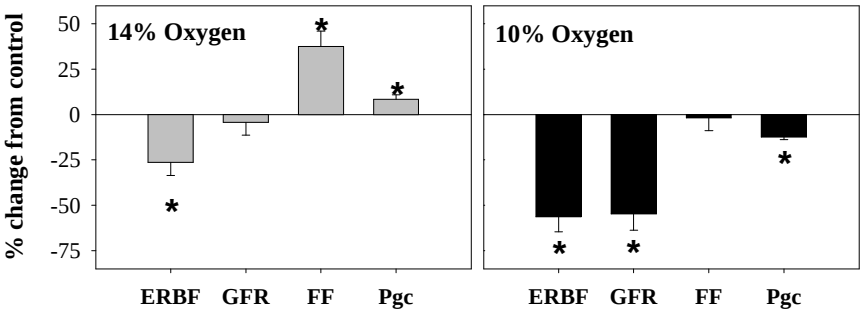
This study investigates the effects of recruitment of nerves on renal segmental resistances. The bursting behaviour of nerve activity can be differentiated into frequency (rate of firing) and amplitude (recruitment of nerves) responses. Increasing levels of hypoxia result in graded increases in renal sympathetic nerve activity (RSNA) amplitude that is associated with progressive renal vasoconstriction (Leonard *et al.*, 2000), but the effects of RSNA on segmental renal vascular resistances are not known. This is of particular interest given our recent identification of two distinct nerve types that are differentially distributed to afferent and efferent arterioles (Luff *et al.*, 1991). Therefore we have estimated glomerular capillary pressure (Pgc) in 3 groups of rabbits exposed to room air (n=6), 14% (n=6) or 10% oxygen (n=8).

Male rabbits (3.0 ± 0.1 kg) were anaesthetised with pentobarbitone (120 mg/kg bolus plus 0.2 mg/kg/min i.v.) and ventilated. Ear artery and vein catheters were placed for the measurement of mean arterial pressure (MAP) and infusion of fluids. Via a flank incision the left kidney was exposed, the renal nerves placed on recording electrodes and the kidney prepared for micropuncture (Denton & Anderson, 1991). Effective renal blood flow (ERBF) and glomerular filtration rate (GFR) were measured via the clearance of ^{14}C para-aminohippurate and ^{3}H-inulin, respectively. Pgc was estimated as stop-flow pressure (Denton & Anderson, 1991). Measurements were made over two 60 min periods before and after exposure to one of the gases. The rabbits were then killed by anaesthetic overdose. Values are mean ± SEM. Data was compared using analysis of variance (*P < 0.05 change from control).

Total RSNA increased in response to 14% O_2 (89 ± 22 %, P < 0.05) and 10% O_2 (162 ± 34 %, P < 0.05). MAP did not change in response to either level of hypoxia. The renal function results are given in the figure. No significant changes in any variable were seen in response to room air. During 14% O_2 ERBF fell but GFR was maintained by an increase in filtration fraction (FF) due to an increase in Pgc indicating preferential post-glomerular vasoconstriction. However, during 10% O_2 there was no change in FF and GFR fell due to a decrease in Pgc indicating a greater pre-glomerular component to the vasoconstriction.



In conclusion, graded reflex activation of RSNA resulted in different patterns of response in renal function, compatible with differential neural control of pre- and post- glomerular resistance.

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INFLUENCE OF RENIN-ANGIOTENSIN SYSTEM BLOCKADE AND DIETARY SODIUM INTAKE ON CARDIOMYOCYTE GROWTH AND CONTRACTILITY

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Angiotensin-II and dietary sodium are known to play important roles in the regulation of myocardial growth (Schmeider *et al.*, 1996). The anti-hypertrophic effects of angiotensin-converting enzyme inhibitors (ACE-I) and angiotensin-II receptor (AT-1) antagonists have been established. Salt restriction has also been shown to reduce cardiac mass experimentally (Morgan *et al.*, 1998). We have previously shown that combined ACE-I and AT-1 antagonist treatment produces a more marked suppression of cardiac growth with reduced dietary Na (Griffiths *et al.*, 1999). This study investigates the alterations in cardiomyocyte excitation-contraction coupling which occur in association with cardiac growth suppression by renin-angiotensin system (RAS) blockade and low Na intake.

Male Sprague-Dawley rats (10-11 weeks) were fed high (4% w/w) or low (0.2%) w/w) Na-containing chow, with tap water for 14 days. From day 7, RAS blockade was achieved by combined daily treatment with ACE-I and an AT-1 antagonist (perindopril 6 mg/kg & losaratan 10 mg/kg, i.p.). At the end of the treatment period, animals were anaesthetized (pentobarbitone sodium, 60 mg/kg, i.p.), hearts removed and isolated ventricular cardiomyocytes prepared by enzymatic dissociation. Compared with the high Na treatment group, RAS blockade combined with Na restriction produced a significant (P<0.05) suppression of cardiac growth evidenced by reduced wet heart weight (1.55±0.04 vs 1.72±0.05 g) and cardiac index (3.92±0.07 vs 4.24±0.11 g/kg). Parallel differences in cardiomyocyte size were observed by determining the mean dimensions of 50 myocytes per animal (n=7 for each group). Myocytes from the low Na group had significantly smaller width (26.6±0.6 vs 29.7±0.06 μm) and length (112.3±2.3 vs 118.9±1.4 μm) compared to the high Na treatment group.

High resolution optical techniques were used to measure the isotonic shortening of single cardiomyocytes under standardised conditions (36°C, 3 Hz stimulation and 1 mM Ca^{2+} Hepes buffer). The basal steady-state contractile performance of myocytes from the low Na treatment group was depressed compared to myocytes from the high Na treatment group (n=50, 51). The maximal cell shortening attained during the contractile cycle (6.40±0.17 vs 7.32±0.16 % resting length) and the maximal rate of shortening (3.85±0.14 vs 4.29±0.11 cell length/s) were significantly reduced, and the contractile cycle time was abbreviated (89.5±2.8 vs 96.8±2.3 ms). Myocytes were exposed to the Na-H exchanger blocker, HOE694 (40 μM, Hoechst Marion Roussel) for 10 min. HOE694 had a significantly greater negative inotropic effect on myocytes from the low Na treatment group. Maximal shortening of myocytes was reduced to 52±2% of initial value in the low Na group (n=8) compared to a reduction to 69±3% of initial level in the high Na group (n=10).

These studies demonstrate that the suppression of cardiac growth which occurs with combined RAS blockade and dietary Na restriction is associated with reduced cardiomyocyte size. Growth suppression is linked with comparatively impaired cardiomyocyte basal contractility, and with increased susceptibility to *in vitro* block of the Na-H exchanger. These responses may represent a functional shift in cardiomyocyte pH homeostasis in the environment of chronically altered angiotensin-II signalling.

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THE ROLE OF ANGIOTENSIN II IN ISCHEMIA AND PRECONDITIONING IN ISOLATED RAT HEART

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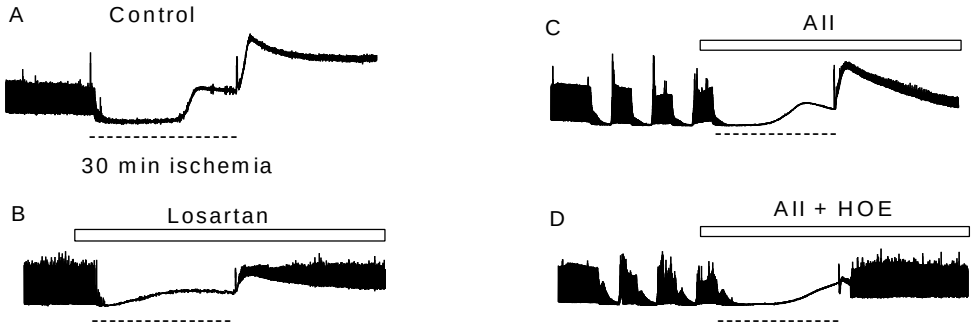
Angiotensin II (AII) antagonists reduce the myocardial damage caused by ischemia and reperfusion (Werrmann & Cohen, 1996) suggesting that AII has a role in causing ischemic damage. It is also known that the inhibition of the cardiac Na^+/H^+ exchanger (NHE1) reduces myocardial damage (Karmazyn *et al.*, 1999) and we recently suggested that ischemic preconditioning was also caused by inhibition of NHE1 (Xiao & Allen, 1999). Another recent discovery is that NHE1 is partially regulated by endogenously released AII (Cingolani *et al.*, 1998). These disparate findings can be explained if AII is a key regulator of NHE1 and if its release is increased after ischemia but reduced after a preconditioned ischemia. We have tested this novel hypothesis.

Rats were anaesthetized with pentobarbitone and the hearts were isolated and developed pressure (DP) was recorded. Some hearts were preconditioned with three cycles of 5 min-ischemia. Reperfusion injury was assessed by DP recovery on reperfusion and by the magnitude of reperfusion contracture (RC).



Panel A shows that 30 min ischemia and reperfusion damaged the heart: the DP recovery was 17 ± 2% of pre-ischemia level and the RC was 65 ± 5 mm Hg (n = 7). Panel B shows that the functional recovery was improved by the application of the AII receptor blocker (losartan; 10 μM): the DP recovery was 55 ± 4% and RC was 35 ± 6 mmHg (n = 6). Preconditioning also improved the functional recovery (not shown): the DP recovery was 72 ± 5% and RC was 25 ± 5 mmHg (n = 6). Panel C shows that when AII (1 μM) was applied to preconditioned heart the preconditioning effect was lost: DP recovery was only 26 ± 4% and the RC was 60 ± 5 mmHg (n = 8). Panel D shows that when AII was simultaneously applied with an NHE1 inhibitor (HOE 642; 10 μM) in preconditioned hearts, the effect of preconditioning was restored: the DP recovery was 75 ± 7% and the RC was 15 ± 3 mm Hg (n = 6).

Thus an AII receptor blocker protects the heart from ischemic damage; AII reverses preconditioning protection and this effect is eliminated by NHE1 inhibition. These results point to a direct role for locally produced AII in regulating NHE1 and in ischemic damage. Direct measurements of AII are needed to confirm this proposal.

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INTRACELLULAR ACIDOSIS PROTECTS AGAINST Ca^{2+} OVERLOAD-INDUCED Ca^{2+} WAVES IN RAT VENTRICULAR MYOCYTES

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In myocytes loaded with the fluorescent Ca^{2+} indicator fluo-3, spontaneous localised release of Ca^{2+} through channels in the sarcoplasmic reticulum (SR), known as a Ca^{2+} spark, can be detected. We have previously shown that acidosis reduces spontaneous Ca^{2+} spark frequency due to inhibition of the SR Ca^{2+} release channel (Balnave & Vaughan-Jones, 1999).

Under conditions in which the SR becomes overloaded with Ca^{2+}, Ca^{2+} sparks summate to produce waves of Ca^{2+} release that propagate throughout the myocyte. In whole hearts Ca^{2+} waves may induce damaging cardiac arrhythmias. Therefore, we investigated whether acidosis can protect myocytes by inhibiting Ca^{2+} wave generation.

Rats were killed by cervical dislocation and ventricular myocytes enzymatically isolated from the hearts and loaded with fluo-3 AM (10 μM). Myocytes were superfused with HEPES-buffered solutions (pH 7.4) at room temperature (~22° C). Ca^{2+} overload was achieved by increasing the Ca^{2+} concentration of the superfusate from 1 mM to 10 mM. An intracellular acid load of ~0.75 pH units was imposed by adding sodium acetate (80 mM) to the superfusate for one minute. A confocal microscope (Leica DM IRBE) was used in line-scan mode to continuously record changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) during a one minute collection period. Images were taken before and one minute after the introduction of the acid load. In a parallel series of experiments carboxy SNARF-1 was used to measure pH_i.

No Ca^{2+} waves were observed in cells prior to the increase in superfusate $[Ca^{2+}]$. When Ca^{2+} overload was induced cells produced Ca^{2+} waves at regular intervals. However, when an acid load was then introduced, Ca^{2+} wave frequency initially decreased, but then gradually increased throughout the collection period to a significantly higher level.

One plausible explanation for the increase in Ca^{2+} wave frequency over time is that the removal of H^+ from the cell via Na^+-H^+ exchange progressively increases the $[Na^+]_i$, which may in turn affect Na^+-Ca^{2+} exchange and cause Ca^{2+} loading at rest and inhibit Ca^{2+} removal during a Ca^{2+} wave. To investigate this hypothesis we added the specific Na^+-H^+ exchange inhibitor HOE 694 (30 μM) to the superfusate. Acidosis in the presence of HOE 694 significantly reduced Ca^{2+} wave frequency and maintained it at a low level throughout the collection period.

The results provide evidence that, during Ca^{2+} overload, intracellular acidosis can be both protective, by directly inhibiting SR Ca^{2+} release, and harmful, by indirectly modifying sarcolemmal Ca^{2+} flux.

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SIMULATION OF STEADY-STATE AND TRANSIENT MECHANICS IN CARDIAC MUSCLE FOLLOWING TROPONIN I PHOSPHORYLATION

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Adrenaline accelerates cardiac mechanics through the PKA mediated phosphorylation of troponin I (Turnbull *et al.*, 1999; Kentish *et al.*, 2000). This acceleration has been reported under conditions of steady activation (Hoh *et al.*, 1988), and in response to an abrupt decrease in [Ca^{++}] (Kentish *et al.*, 2000). It is of interest to see whether a model that can simulate acceleration of mechanics under steady activation (Rossmanith &Tjokorda, 1998) is also sufficient to simulate the accelerated relaxation of tension initiated by an abrupt decrease in [Ca^{++}].

Cross-bridge kinetics under steady activation have been probed by means of measurements of dynamic stiffness initiated by small-amplitude changes to muscle length, and in particular by the frequency, fmin, of the length-change that results in minimum stiffness. Fmin has been shown to increase by approximately 50% in response to adrenaline. Kentish *et al.* (2000) reported that the tension relaxation following flash photolysis of diazo-2 is well fitted by a bi-exponential decay, and that the acceleration in relaxation occurs principally because of an increase in the slow rate constant with the fast rate constant being only minimally affected.

For steady activation, the change in fmin can be simulated by increasing the rate of detachment of cross-bridges from the force-producing state. If the abrupt decrease in [Ca^{++}] is modeled by simply preventing cross-bridges from reattaching, relaxation of tension occurs. It was found that this relaxation profile matched the experimental data: it consisted of two exponential processes with 'slow and 'fast' rate constants; the increase in cross-bridge detachment required to simulate the shift in fmin resulted in speeding up of the slow exponential process whilst only slightly changing the fast component. However the amplitudes of these exponential processes were not well fitted. In particular, the amplitude of the fast exponential process was too small, leading to an overall slower relaxation of tension than reported. What was not included in this simple model were factors like calcium kinetics induced by an abrupt decrease in [Ca^{++}], or cooperativity between cross-bridges, and these factors could well play a part in determining the profile of tension relaxation.

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EFFECTS OF SUBSTRATES AND INSULIN ON THE EFFICIENCY OF RAT PAPILLARY MUSCLES

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It has been reported recently in working rat heart preparations that the provision of insulin or the addition of ketones to physiological buffer containing 10 mM glucose increases mechanical efficiency by increasing the work output per beat (Sato *et al.*, 1995). There are also conflicting reports as to whether propionyl-L-carnitine (PLC) can improve mechanical efficiency.

We decided to take advantage of the maintained mechanical performance of isolated papillary muscles to test the hypothesis that the provision of certain substrates and insulin can alter mechanical contractility and efficiency. Papillary muscles were dissected from the left ventricle of adult rats that had been killed humanely. The muscles were mounted on a thermopile containing antimony-bismuth thermocouples to measure heat production (Baxi *et al.*, 2000) and were made to work against a range of different afterloads. All experiments were carried out at 27°C, with preparations being stimulated in trains of 10 contractions at 0.2 Hz at different afterloads. The resting (non-beating) metabolism of the hearts were measured and the net mechanical efficiency and the contractile efficiency, see Suga (1990) were measured. There were 4 substrate combinations (i) 10 mM glucose, (ii) 10 mM glucose + 100 nM insulin, (iii) 10 mM glucose + 4 mM D-β-hydroxybutyrate + 1 mM acetoacetate, and (iv) 10 mM glucose + 2mM propionyl-L-carnitine. A random allocation of the different substrate combinations was made using a Latin square design. Analysis of variance was performed on the data to test for differences between the various solutions and differences were considered statistically significant at $P<0.05$.

The resting heat (basal metabolism) declines exponentially with time (22% over 4-6 h) but none of the substrates altered its magnitude. The work per beat, the enthalpy and the mechanical efficiency were essentially unchanged in the four substrates ($P>0.05$). Maximum work output occurred with afterloads in the 0.4 to 0.6 P/Po range, where Po = isometric force. The mean work per beat was close to 1.1 mJ g^{-1}. A force-length-area (FLA) analysis of the isotonic data showed that contractile efficiency was also unchanged ($P>0.05$).

There are obviously clear energetic differences between the effects of different substrates in buffer-perfused isolated hearts working at 5 Hz and 37°C and papillary muscles working at 0.2 Hz and 27°C.

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REGULATION OF CARBOHYDRATE OXIDATION DURING EXERCISE AND HEAT STRESS

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Muscle glycogenolysis and carbohydrate oxidation are both increased by heat stress during prolonged exercise (Febbraio *et al.*, 1994). Glycogen phosphorylase and pyruvate dehydrogenase, the key enzymes which regulate glycolytic flux and carbohydrate oxidation respectively, are maximally activated 1 min after the onset of moderate intensity exercise (Howlett *et al.*, 1998). Thus, the aim of this study was to examine the effect of heat stress on muscle metabolism at the onset of moderate intensity exercise.

Six active men (26.0 ± 5.0 yrs, 75.0 ± 6.7 kg, VO_2 peak = 3.55 ± 0.39 l min^{-1}, mean ± SD) reported to the laboratory on two occasions in the morning after an overnight fast. Subjects positioned a rectal thermometer, a basal blood sample was obtained after a catheter was positioned in the antecubital space of one arm, and a resting muscle temperature was obtained from the vastus lateralis. After this time, subjects entered an environmental chamber set at either 20°C (CT) or 40°C (HT) (relative humidity 35% for both trials) and rested for 20 min, before a pre-exercise blood sample, rectal (T_{rec}) and muscle (T_{mus}) temperature measures were made and a muscle biopsy was obtained. Subjects then commenced cycling exercise for 20 min at a power output eliciting ~70% VO_2 peak. Muscle biopsies were obtained at 1 min and 5 min of exercise. Together with the pre-exercise sample, these were analysed for adenosine-5'-triphosphate (ATP), creatine (C), phosphocreatine (PCr), glucose-6-phosphate (G-6-P), pyruvate (pyr), lactate (mLa^-) and acetyl coA (ACoA). Muscle glycogen (Gly) was measured pre-exercise and at 5 min. In addition to those obtained at rest, blood samples were obtained at 5 min intervals throughout exercise for subsequent measurement of plasma adrenaline (Adr). T_{mus} and T_{rec} were also measured at 1, 5 and 20 min of exercise. Although T_{mus} was similar at rest, 20 min of passive exposure to heat increased (P<0.05) T_{mus} in HT compared with CT. This difference was maintained throughout exercise. Neither T_{rec} nor Adr were different at rest, pre-exercise or at 1 and 5 min but T_{rec} was higher (P<0.05) and Adr tended to be higher (P=0.051) after 20 min of exercise in HT compared with CT. In addition, neither ATP nor Pyr were affected by treatment or exercise. Although C, G-6-P, mLa^- and ACoA increased (P<0.05) and PCr and Gly decreased (P<0.05) when comparing concentrations at 1 and 5 min with those pre-exercise, no differences were observed when comparing HT with CT.

The results of the present study demonstrate that heat stress does not alter intramuscular metabolism at the onset of exercise. It is likely, therefore, that as exercise in the heat progresses and the difference in circulating adrenaline is augmented, the activation of the enzymes which regulate carbohydrate utilisation are increased resulting in enhanced glycogen use.

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EFFECT OF SIMULATED MODERATE ALTITUDE EXPOSURE ON SKELETAL MUSCLE METABOLITES AND pH REGULATION IN HIGHLY TRAINED ATHLETES

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Athletes commonly use altitude exposure in an attempt to enhance aerobic exercise performance. The aims of this study were to investigate whether hypoxic exposure would in fact increase muscle anaerobic metabolism, H^+ buffering capacity, improve exercise acid-base regulation and exercise performance. Six well-trained athletes slept by night (mean 9.5 h night^{-1}) at simulated moderate altitude (3000m, F_IO_2 = 15.48 %) for 23 nights (Live High & Train Low, LHTL) and lived and trained by day in Canberra (altitude ~600 m). 7 control athletes lived, trained and slept in Canberra (C). Subjects completed an exercise trial comprising 4 × 4 min submaximal workrates, 4 min rest and 2 min at 5.6 W kg^{-1}, 4 d before and 3 d after LHTL. A vastus lateralis muscle biopsy was taken at rest and after exercise and analysed for *in-vitro* H^+ buffering capacity (βm), [H^+], and glycogen, lactate, ATP, PCr and Cr contents. $\dot{V}O_{2peak}$ was measured 5 d before during d 11 and 2 d after LHTL, where the subjects completed the above exercise test with an additional 2min, completing as much work (kJ_{2min}) as possible. Total work (kJ_{2min}) was maintained after LHTL, although $\dot{V}O_{2PEAK}$ was reduced (7%, P<0.05). The submaximal efficiency of LHTL group was improved 0.8% from PRE (18.9 ±2.7%) to POST (19.7 ±2.4%), (p=0.007). Lactate post exercise tended to be lower after LHTL compared with C (p= 0.06). LHTL did not modify muscle [H^+], or other metabolites at rest or at fatigue. However, resting muscle ßm was increased by 17.5 ± 4.9% after LHTL (see Table).

		PRE LHTL	POST LHTL	PRE CONT	POST CONT
$\dot{V}O_{2peak}$ (l min^{-1})	EX	5.08±0.34	4.78±0.36*	4.95±0.45	4.87±0.44
Total work (kJ_{2min})	EX	50.0±4.2	49.2±4.2	50.5±6.0	50.3±5.8
[H^+] (nmol L^{-1})	RE	71.1±3.8	67.3±5.5	69.0±4.4	66.4±4.8
	EX	156.6±22.8	139.9±20.4	162.3±21.9	140.5±20.5
βm (µmol H^+ g dw^{-1} pH^{-1})	RE	138.4±13.3*	162.7±14.2*	145.6±14.8	145.7±8.8
ATP (mmol kg dw^{-1})	RE	28.6±0.8	28.6±0.6	28.7±1.2	28.7±1.0
	EX	18.0±0.5	18.1±0.5	18.0±0.3	17.9±0.4
PCr (mmol kg dw^{-1})	RE	87.9±0.7	87.9±0.5	89.1±1.9	89.0±1.9
	EX	62.5±0.9	62.3±0.7	62.9±0.7	63.0±1.1
Glycogen (glucosyl units) (mmol kg dw^{-1})	RE	576±98	571±79	611±68	599±56
	EX	245±15	247±10	235±19	227±27
lactate (mmol kg^{-1})	RE	5.9±2.9	6.0±2.5	4.9±0.6	4.9±0.5
	EX	44.5±7.1	40.1±12.1	47.5±2.5	48.2±3.4
Anaerobic ATP Production Rate (mmol kg dw s^{-1})		0.57±0.08	0.51±0.16	0.62±0.03	0.63±0.05
Protein (mg mg muscle^{-1})	RE	0.177±0.010	0.174±0.013	0.168±0.014	0.166±0.015
	EX	0.175±0.013	0.174±0.012	0.168±0.012	0.166±0.014

Data are mean ± SD, Post>Pre * P<0.05

In conclusion, LHTL for 23 nights at 3000m did not enhance muscle anaerobic metabolism, despite an increase in mechanical efficiency and a decrease in $\dot{V}O_2$ during exercise., Despite the increase in muscle ßm, muscle pH regulation was not improved. This increase in ßm with hypoxic exposure may be related to some other function than pH regulation.

PROGRESSIVE INCREASE IN HUMAN SKELETAL MUSCLE AMPK a2 ACTIVITY AND ACETYL-CoA CARBOXYLASE PHOSPHORYLATION BY AMPK DURING PROLONGED EXERCISE

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Activation of AMP-dependent protein kinase (AMPK) is a key regulatory step involved with stimulation of contraction mediated glucose uptake and fat oxidation during exercise. Sprint exercise (30 sec all-out) doubles skeletal muscle AMPK α1 and α2 activity and phosphorylation of nNOSμ at Ser-1451, was increased by approximately 5.5-fold (Chen *et al.*, 2000). NO has been shown to increase glucose uptake during exercise/contraction. The sprint exercise increased phosphorylation of acetyl-CoA carboxylase (ACC_β) at Ser-79 (AMPK phosphorylation site, inhibitory) by approximately 8.5-fold (Chen *et al.*, 2000). Phosphorylation of ACC_β at Ser-79 in rats has been shown to increase fat oxidation.

The aim of this study was to examine AMPK (α1 and α2) activity, nNOS and ACC_β phosphorylation during moderate exercise that is associated with higher rates of skeletal muscle glucose uptake and fat oxidation than sprint exercise. Seven active healthy males completed 30 min of cycling at a workload requiring 63 ± 1% of VO_2 max. Muscle biopsies were obtained from the vastus lateralis muscle at rest and after 5 and 30 minutes of exercise. These samples were analysed for AMPK α1 and α2 activity, ACC_β phosphorylation (at Ser-79) and nNOSμ phosphorylation (at Ser-1451).

AMPK α1 activity was not altered by moderate exercise, however, AMPK α2 activity was significantly (P<0.05) elevated after 5 min (~2 fold compared with rest) of exercise and further elevated after 30 min of exercise (~3 fold compared with rest). There was no significant change in nNOS phosphorylation (P=0.16) after 5 min or 30 min of exercise. ACC_β phosphorylation was increased (P<0.05) after 5 min (~18 fold compared with rest) and increased further after 30 min (~36 fold compared with rest). In addition, there tended to be a progressive increase in fat oxidation (P=0.06) during exercise. Previous studies have demonstrated a progressive increase in glucose uptake during similar exercise.

In summary, moderate exercise causes a progressive increase in AMPK α2 activity, ACC_β phosphorylation and fat oxidation. This supports the hypothesis that AMPK regulates skeletal muscle fat oxidation during exercise by phosphorylating and inhibiting ACC_β. The results also suggest that AMPK α2 and AMPK α1 are activated differently depending on the exercise intensity since both isoforms of AMPK are activated during maximal sprint exercise. nNOS phosphorylation was not increased when AMPK α2 activity was elevated suggesting that both AMPK α1 and α2 are required for maximal phosphorylation of nNOS.

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EFFECT OF PRIOR EXERCISE ON GLUCOSE METABOLISM IN TRAINED MEN

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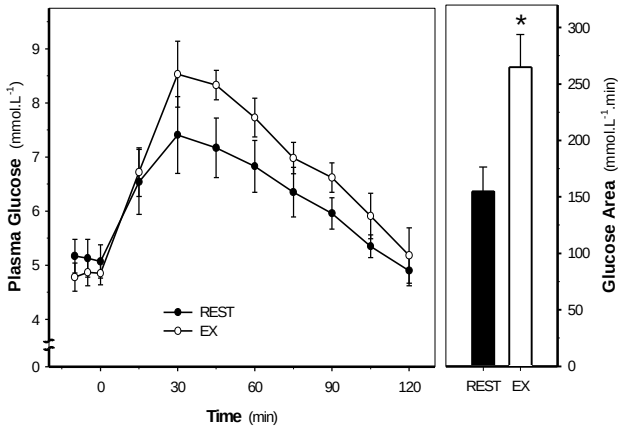
Previous studies have demonstrated that oral glucose tolerance is impaired in the immediate period after exercise compared with 24 hr postexercise (Bonen *et al.*, 1998; King *et al.*, 1995). Further, the insulinaemic response to oral glucose is greater (King *et al.*, 1995) or not different (Bonen *et al.*, 1998) after exercise. Taken together, these results indicate a relative whole-body insulin resistance in the immediate period after exercise. Using a double tracer technique (Steele *et al.*, 1968) glucose kinetics were examined during a 75g, 2 h oral glucose tolerance test (OGTT) 30 min postexercise (EX; 60 min, 71 ± 2% VO_{2peak}, mean ± SEM) and at rest (REST) in six physically trained men (29.2 ± 4.9 yr, 23.2 ± 1.2 kg.m^{-2} BMI, 65.4 ± 6.9 mL kg^{-1} min^{-1} VO_{2peak}; mean ± SD).



As shown in the Figure, the integrated area under the plasma glucose curve was 102 ± 35% greater in EX versus REST (P=0.011). The higher glucose response occurred even though whole-body glucose R_d was 24% higher after exercise (P=0.044, main effect). Whole body R_a was 25% higher after exercise (P=0.033, main effect). There were no differences in total (2 h) endogenous glucose appearance (R_aE) or magnitude of suppression of R_aE despite a higher R_aE from 15-30 min of the OGTT in EX. However, cumulative oral glucose appearance was 30% higher in EX (P=0.030, main effect).

There were no differences in glucose clearance rate, or plasma insulin and lactate responses between the two conditions. Plasma NEFA were significantly higher 30 min postexercise, however, were suppressed to similar levels during the OGTT.

The greater appearance of oral glucose after exercise may be a result of a higher intestinal absorption rate and/or reduced glucose uptake by the liver. Similar to other studies (Hamilton *et al.*, 1996; Mæhlum *et al.*, 1978) these results suggest that adaptations in splanchnic tissues by prior exercise facilitate greater glucose output from the splanchnic region following glucose ingestion, thereby mediating a greater glycaemic response and consequently, greater systemic glucose uptake.

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EFFECT OF ACUTE EXERCISE ON HSP72 EXPRESSION IN HUMANS

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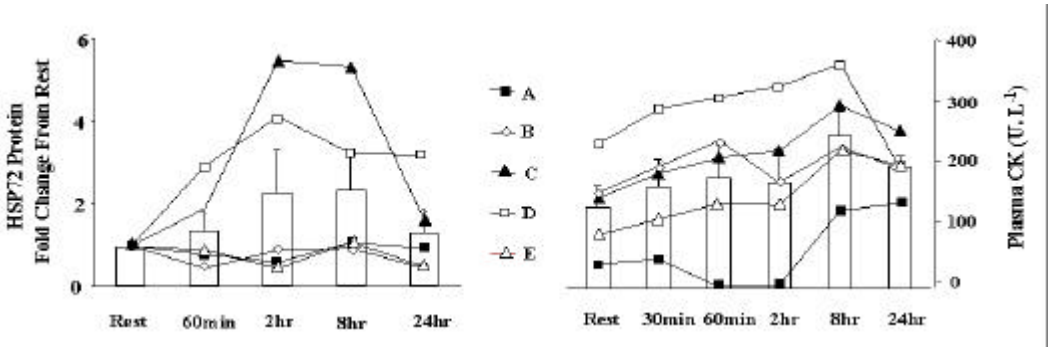
Increased expression of the highly inducible Stress Protein HSP72, protects cells from harmful stimuli. Acute exercise has been observed to elevate HSP72 gene and protein expression in rodent skeletal muscle within 3 hours of exercise (Salo *et al.*, 1991). However, whilst we have recently demonstrated that acute exercise increases HSP72 gene expression (Febbraio & Koukoulas, 2000), the time-course for increases in HSP72 protein after a single bout of exercise has not been well characterized. Extracellular HSP72 has been implicated in immune function, and although exercise is a known stimulus for the HSP72 response, it is not known whether acute exercise increases HSP72 in the circulation. Hence, the aims of the present study were to examine a) the time-course for gene transcription to protein expression and b) whether exercise increases HSP72 in the circulation.

Five active, but not specifically trained, males (26.4 ± 4.1 years, 75.2± 8.7 kg, VO_{2peak} = 3.6 ± 0.38 l min^{-1}, mean ± SD) ran on a treadmill for 60 min at a workload corresponding to 70% VO_{2peak} (0% grade). Muscle biopsy samples were obtained from the *vastus lateralis* at rest (R), immediately post-exercise (60 min), 2, 8, and 24 hours after exercise (2,8,24 h). In addition, at these time points and at 30 min during exercise (30 min) blood was sampled from a forearm vein. Muscle HSP72 gene expression was determined using real-time Polymerase Chain Reaction (rt-PCR). HSP72 protein expression in muscle tissue and serum samples was determined by an enzyme-linked immunosorbent assay. Plasma creatine kinase (CK) was measured and used as a marker of muscle damage. HSP72 mRNA expression was elevated 6.5 fold (P<0.05) from rest within 2 hours of exercise, but HSP72 protein expression was not significantly elevated compared with rest. However, the HSP72 protein expression was variable since two subjects demonstrated between 1.9 and 5.4 fold increases in HSP72 protein at the completion of exercise, and at 2 and 8 h after exercise (see Figure). Of note these subjects displayed the highest levels of plasma CK post exercise (see Figure). Serum HSP72 protein increased from rest, both during and after exercise (0.13 ± 0.10 vs 0.87 ± 0.24 and 1.02 ± 0.41 ng mL^{-1} at rest, 30 min and 60 min respectively, P<0.05, mean ±SE).



The results indicate that whilst exercise increased HSP72 gene expression in skeletal muscle within 2 hours, this was insufficient to induce a significant elevation in HSP72 protein expression. This suggests that HSP72 protein synthesis is dependent upon post-transcriptional regulation. Nonetheless, increases in HSP72 protein expression at the completion of exercise in two subjects, indicate that HSP72 protein synthesis has a rapid time-course when acute exercise causes damage to the cell membrane structure. The novel observation that blood-borne HSP72 is elevated following acute exercise warrants further investigation of the role of HSP72 in the interaction between exercise and immune function.

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AMPK SIGNALING DURING EXERCISE IN HUMAN SKELETAL MUSCLE; ACETYL-CoA CARBOXYLASE AND nNOS PHOSPHORYLATION

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AMP-activated protein kinase (AMPK) is a metabolic stress sensing protein kinase responsible for coordinating metabolism to energy demand. Recently, AMPK and nitric oxide (NO) have been implicated in the regulation of muscle glucose uptake during exercise (Bradley, Kingwell & McConell, 1999, Hayashi *et al.*, 1998). AMPK phosphorylates and activates eNOS in ischemic rat heart (Chen *et al.*, 1999) and exercise increases rat skeletal muscle NOS activity. It is not known if AMPK phosphorylates nNOSμ. In addition, it has been shown in rats that AMPK regulates fat oxidation in muscle by phosphorylating and inhibiting acetyl-CoA-carboxylase (ACC). This study examined the effect of intense exercise in humans on skeletal muscle AMPK activity and phosphorylation, by AMPK, of ACC_β and nNOSμ.

Eleven healthy, young individuals (seven males, four females; 22 ± 1.6 yr, 73.8 ± 4.8 kg, VO_2 peak: 3.0 ± 0.28 l min^{-1} (mean ± SEM) completed a 30 second maximal intensity cycling bout. A muscle biopsy sample was obtained from the vastus lateralis m. before and immediately after exercise. The muscle samples were analysed for AMPK activity, ACC_β phosphorylation (at Ser-79) and nNOSμ phosphorylation (at Ser-1451) and muscle metabolites.

A significant energy imbalance existed during the 30 second sprint as indicated by significant (P<0.05) falls in muscle ATP and creatine phosphate (PCr) and increases (P <0.05) in IMP. Muscle lactate increased (P <0.05) while glycogen decreased (P <0.05) from rest to post exercise. The calculated skeletal muscle free ADP and free AMP increased (P <0.05) following exercise. Both α1 and α2 AMPK activity increased approx. 2-fold during exercise. Exercise also increased phosphorylation of nNOSμ at Ser-1451 (AMPK phosphorylation site) by approximately 5.5-fold. Whereas phosphorylation of eNOS at Ser-1177 is associated with increased NOS activity (Chen *et al.*, 1999) we do not yet know if phosphorylation of nNOSμ at Ser-1451 has the same effect. Exercise increased phosphorylation of ACC_β at Ser-79 (AMPK phosphorylation site) by approximately 8.5-fold. Phosphorylation of ACC_β at Ser-79 has been shown to reduce ACC activity.

These results indicate that skeletal muscle α1 and α2 AMPK activity increases during maximal exercise in humans and that AMPK may exert effects on metabolism via phosphorylation of ACC_β and nNOSμ. The increase in ACC_β phosphorylation was surprisingly large given that anaerobic metabolism predominates during sprint exercise therefore the contribution of fat oxidation is small.

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EFFECT OF PROLONGED EXERCISE AND GLYCOGEN AVAILABILITY ON HSP72 PRODUCTION IN CONTRACTING HUMAN SKELETAL MUSCLE

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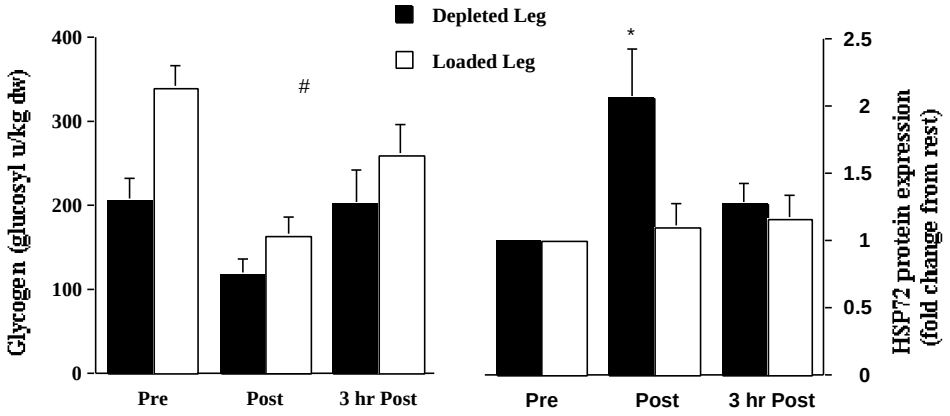
Physical exercise increases heat shock protein (HSP) 72, the inducible form of the 70 kDa family of HSP, in a variety of tissues in several mammalian species. The exact perturbation/s caused by exercise that induce HSP is/are unknown and not easily determined because many known stimuli of HSP increase simultaneously during exercise. Of note, however, HSPs require ADP as a cofactor for peptide binding, and have a low binding affinity in the presence of ATP. It has been suggested, therefore, that low energy states increase HSP-peptide binding, which decreases the free pool of HSPs, resulting in increased HSP production (Moseley, 2000). We have recently demonstrated that HSP72 gene expression increased late in exercise when intramuscular glycogen content was reduced to low levels (Febbraio & Koukoulas, 2000). Since HSP expression is influenced by the energy status of the cell, we hypothesized that the low glycogen levels late in exercise may have provided the stimulus for the HSP response.

To test this hypothesis, seven physically active, but not specifically trained men, [mean age 26 (range 19-33), and height 1.84 m (range 1.75–1.96)] participated in the study. Each subject performed 1 h of single-legged cycling, followed by 1 h of double-armed cranking (in order to deplete muscle glycogen in one leg), 16 h before performing 5 h of two-legged knee extensor exercise at 40%$W_{max,leg}$. Subjects then rested for a further 3 h. Muscle biopsies were obtained before (Pre), immediately after (Post) and three hours into recovery (3 h Post) from exercise. These samples were analysed for muscle glycogen, HSP72 gene and protein expression. In addition, catheters were placed in one femoral artery and both femoral veins and blood was sampled from these catheters prior to exercise and at 1 h intervals during exercise and into recovery for the measurement of arterial-venous differences in serum HSP72. Plasma creatine kinase (CK) was also measured from arterial blood samples. The exercise bout 16 h before the experimental trial was effective in reducing resting glycogen content by 40% (see Figure) and this difference was maintained throughout exercise (P<0.05; main treatment effect). Neither HSP72 gene or protein expression were different Pre-exercise. However, both HSP72 gene and protein increased (P<0.05) after 5 h of exercise in the glycogen depleted leg, but not in the leg with normal pre-exercise glycogen content (see Figure). Exercise did not increase plasma CK concentrations and we were unable to detect HSP72 in the serum of any samples.



These results demonstrate that while acute concentric exercise is capable of increasing HSP72 in human skeletal muscle, it does so only when glycogen is reduced to relatively low levels. Hence, our data provide clear evidence that HSP72 protein expression is related to glycogen availability. In addition, because CK did not increase and we found no evidence of HSP72 in the femoral venous effluent, intact muscle membranes appear to be impermeable to HSP72.

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RESISTANCE TRAINING AND SKELETAL MUSCLE OXIDATIVE CAPACITY IN CHRONIC HEART FAILURE

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In chronic heart failure (CHF) peripheral factors, including skeletal muscle atrophy and deconditioning, rather than factors related to cardiac performance are the major cause of exercise limitation (Clark *et al.*, 1992). Aerobic exercise training has been shown to result in elevated VO_{2peak} and mitochondrial volume density in these patients (Belardinelli *et al.*, 1997). We examined the effects of resistance exercise training on VO_{2peak}, oxidative capacity and capillary to fibre ratio in six patients with stable CHF (age = 68 ± 8 years; left ventricular ejection fraction = 24 ± 8%, mean ± SD). Resting muscle biopsies were taken from the vastus lateralis prior to and following an 11 week resistance training protocol. Muscle samples were analysed for mitochondrial ATP production rate (MAPR), citrate synthase (CS) and capillary density. A graded cycle ergometer test was used to determine peak oxygen uptake ($VO_{2\ peak}$).

Results are shown in the table. CS activity increased (P = 0.02) in response to the training stimulus. There were also trends towards increases in MAPR expressed per kilogram of muscle in the presence of two of the five substrate combinations. A trend towards an increase in capillary to fibre ratio was also found (P = 0.08) which might eventually be reflected in an increase in VO_{2peak} (P = 0.12).

Muscle Data	**Baseline**	**Endpoint**	***P***
MAPR (mmol min^{-1} kg^{-1})			
P&M	3.71 ± 0.89	5.16 ± 1.08	0.10
PC&M	1.70 ± 0.52	2.05 ± 0.28	0.29
α – KG	3.09 ± 1.08	3.99 ± 0.68	0.11
S&R	3.02 ± 1.09	4.08 ± 0.71	0.32
PPKM	4.54 ± 1.02	4.90 ± 0.87	0.64
Citrate Synthase	13.1 ± 2.6	17.8 ± 1.9	0.02
Capillary/fibre ratio	1.00 ± 0.01	1.18 ± 0.05	0.08
VO_{2peak} (ml kg^{-1} min^{-1})	13.5 ± 1.0	15.8 ± 0.4	0.12

Data presented as mean ± SEM. P = pyruvate; M = malate; PC = palmitoyl-carnitine; α-KG = α ketoglutarate; S = succinate; R = rotenone. (n = 6 except Capillary/fibre ratio n = 3; VO_{2peak} n = 5).

In conclusion, there was a significant increase in CS activity following eleven weeks of resistance training. If this form of training also results in reversal of muscle atrophy, it would be a most appropriate treatment modality for CHF patients.

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INHIBITION OF NITROXYL-MEDIATED RELAXATIONS IN RAT AORTA BY FREE RADICAL NO-INACTIVATING AGENTS IS CAUSED BY THE OXIDATION OF NITROXYL INTO FREE RADICAL NO

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Angeli's salt undergoes dissociation in neutral solution to form nitroxyl (NO^-) and nitrite anions (Pino & Feelisch, 1994). The nitroxyl anion appears to be a physiologically relevant redox species of nitric oxide (NO) as there is abundant evidence to suggest that it may be formed endogenously (Hughes, 1999). Many studies have compared the activity of authentic NO in the presence of substances that are known to affect the activity of the free radical form of NO, such as carboxy-PTIO, hydroxocobalamin and superoxide anions. However, limited information is available on how these agents affect other redox forms of NO such as NO^-. Therefore, the aim of this study was to investigate the effect of these agents on relaxations to Angeli's salt in the rat aorta and to determine the mechanism involved.

Briefly, the aorta, removed from male Sprague-Dawley rats, killed by CO_2 asphyxiation, and mounted in 8 ml organ baths, was contracted with phenylephrine (1 μM). Relaxations were produced to Angeli's salt (0.3 μM) and compared in the presence and absence of the free radical NO scavenger, carboxy-PTIO (100 μM), the NO chelator, hydroxocobalamin (30 μM) and the superoxide generator, pyrogallol (30 μM).

Angeli's salt produced relaxations that were on average 62.2 ± 4.4 % (n = 22) of phenylephrine-induced tone. Carboxy-PTIO (100 μM), potently inhibited Angeli's salt-induced relaxations in the aorta (35.9 ± 9.7% of phenylephrine-induced tone, n = 5, paired t-test, $P < 0.05$). Similarly, relaxations to Angeli's salt were abolished by hydroxocobalamin (30 μM) in the aorta (5.7 ±2.5%, n = 4, paired t-test, $P < 0.05$). The superoxide generator, pyrogallol (30 μM) also significantly reduced relaxations to Angeli's salt in the aorta (28.8 ± 7.4 %, n = 5, paired t-test, $P < 0.05$). The most likely explanation for the inhibitory effect of these agents on Angeli's salt-mediated relaxations is that upon addition, the NO^- from Angeli's salt is oxidised to free radical NO, hence making it susceptible to these inactivating agents. This effect was further illustrated by testing the levels of NO generated by Angeli's salt using a free radical NO selective sensor. Angeli's salt was added to solution containing $CuSO_4$, with Cu (II) acting as an oxidising agent. Under these conditions NO release from Angeli's salt (0.3 μM) was 185.1 ± 13.6 nM (n = 4) and in the presence of carboxy-PTIO, hydroxocobalamin and pyrogallol the level of free NO was significantly decreased.

These results indicate that NO^- generated from Angeli's salt can easily undergo oxidation to form free radical NO in the rat aorta possibly through an unidentified cellular source. This may explain the inhibition of NO^- mediated relaxations by carboxy-PTIO, hydroxocobalamin and superoxide which are regarded as being specific for targeting the free radical form of NO.

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COMPARISON OF ENDOTHELIAL CELL FUNCTION IN LARGE AND SMALL ARTERIES FROM THE OBESE ZUCKER RAT

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The genetically obese (fa/fa) Zucker rat (OZR) is well established as an animal model of insulin resistance and Type II diabetes, which are conditions associated with the development of severe vascular complications including coronary heart disease and nephropathy. However there have not been extensive pharmacological studies examining vascular function in this model. The aim of the present study was to compare endothelial function, as well as various metabolic parameters, between the OZR and the corresponding lean littermate control Zucker rat (LZR) in both a large and small artery preparation.

At 24-25 weeks of age, the female OZR showed significantly (P<0.05, Student's t-test) increased non-fasting blood glucose levels (6.7 ± 0.4 mM, n=22), reduced glucose tolerance (11.4 ± 0.5 mM peak blood glucose after 2.5 g/kg oral glucose load, n=9), elevated blood pressure (166 ± 4 mm Hg, n=12) and increased excretion of urinary protein (63.4 ± 6.7 mg/day, n=10), compared to the corresponding female littermate LZR (5.6 ± 0.2 mM, n=25; 8.5 ± 0.6 mM, n=9; 142 ± 6 mmHg, n=11; 27.6 ± 2.5 mg/day, n=9; respectively). The 24-25 week-old rats were killed by CO_2 asphyxiation. Rings of endothelium-intact thoracic aortae and renal arteries (internal diameter 400-600 μm) were mounted in organ baths and the Mulvany small vessel myograph, respectively, for isometric force measurements. Renal artery experiments were carried out in the presence of 10 μM indomethacin to exclude the involvement of prostanoids. Aortae and renal arteries were constricted with noradrenaline (0.1 μM) and phenylephrine (1-3 μM) respectively before obtaining endothelium-dependent relaxant responses to acetylcholine (ACh, 1 nM – 10 μM) or endothelium-independent relaxations to sodium nitroprusside (SNP, 0.1 nM – 10 μM).

Relaxations to ACh were significantly less (P<0.05, 2-way MANOVA) in aortae from the OZR than from the LZR, with no change in sensitivity but a 31% reduction (P<0.05, Student's t-test) in maximum response (OZR: 60 ± 6 % of precontraction, n=8; LZR: 87 ± 4 %, n=8). In contrast, in the renal artery the maximum response to ACh was not different in vessels from the OZR (76 ± 5 %, n=7) and LZR (71 ± 5 %, n=7), but log concentration-response curves to ACh were slightly shifted to the right by 2.4-fold (95% CL = 1.9, 2.9) in the OZR. Responses to SNP were not significantly different (P>0.05, 2-way MANOVA) between the LZR and OZR for either vascular tissue. In aortic rings, the nitric oxide (NO) synthase inhibitor nitro-L-arginine (L-NAME, 100 μM) abolished relaxant responses to ACh without affecting those to SNP, demonstrating that NO is solely responsible for the endothelium-dependent vasodilator responses to ACh. Responses to ACh in renal arteries were only partially inhibited by L-NAME; this inhibition was more pronounced in renal arteries taken from the OZR (E_{max} 21 ± 5 %, n=7) than from the LZR (E_{max} 48 ± 10 %, n=7). The remaining relaxation in the presence of L-NAME is presumably due to the release of endothelium-derived hyperpolarising factor (EDHF).

The findings demonstrate that the female OZR at 24-25 weeks of age displays many of the characteristics of human NIDDM, including glucose intolerance, moderate hypertension, mild hyperglycemia and proteinuria. The results suggest that the OZR exhibits endothelial dysfunction in both the large (aorta) and small (renal) arteries studied, but the nature of the dysfunction differs. In the aorta, endothelium-derived NO is defective, whereas in the renal artery it appears that the response to EDHF but not to NO is impaired in the OZR.

HIGH GLUCOSE INDUCED APOPTOTIC AND HYPERTROPHIC GROWTH RESPONSES IN HUMAN ENDOTHELIAL CELLS

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High ambient glucose concentration has been correlated with the development of diabetic microangiopathy. Hence the aim of this study was to assess the effects of high glucose on both endothelial cell growth and the induction of apoptosis.

Cultures of subconfluent Human Umbilical Vein Endothelial Cells (HUVECs) were exposed to 5 mM D-glucose, 25 mM D-glucose or 5 mM D-glucose plus 20 mM mannitol (osmotic control) over a 24, 48 or 72 hour period. Cell growth was assessed using cell number, protein content and thymidine uptake (n=5). Apoptosis was assessed morphologically by DAPI staining and necrosis was assessed by measuring LDH release (n=3). Results were standardised to those obtained from culture in 5 mM glucose.

At 24 hours, 25 mM glucose caused an initial proliferative response with an increase in cell number to 123.7±2.8% (mean± SEM) (P<0.05), a response not observed with the mannitol, the osmotic control 97.4 ± 4.1%. Thymidine uptake per cell was significantly reduced following exposure to 25 mM glucose 51.2±1.2% (P<0.01) but not mannitol 109.4±12.2%. Cell protein content did not change with either treatment.

At 72 hours, a marked reduction in cell number was observed in 25mM glucose (65.7± 1.8%; P<0.05). A similar effect was observed with 20 mM mannitol (69.4 ± 1.9%; P<0.05. Conversely, the protein uptake per cell increased by 160.7±4.6% in 25 mM glucose and 165.8 ± 4.1% in 20 mM mannitol. At 72 hours, thymidine uptake per cell returned to control levels (101.25±3.725% in 25 mM glucose and 96.8±4.15% in 20 mM mannitol). The increase in cell protein and reduction in thymidine uptake at 72 hours is indicative of a hypertrophic response of the cells to these conditions.

Due to the differential effects of 25 mM glucose and 20 mM mannitol on the early growth parameters, we investigated whether the reductions in cell number seen at 72 hours could be due to different modes of cell death. It has previously been suggested that both glucose and mannitol enhance apoptosis in HUVECs. Exposure of HUVECs to 25 mM glucose resulted in a significant increase in the apoptotic index of 48.2±1.2%; P<0.01 at 72 hours, an effect not observed with 20 mM mannitol. In contrast, exposure to 20 mM mannitol for 72 hours lead to a significant increase (13±1%; P<0.05) in LDH levels, an effect not observed with 25 mM glucose.

Prolonged exposure of HUVECs to high glucose results in an initial proliferative response followed by a decrease in cell number due to apoptosis and subsequent compensatory cell hypertrophy. This effect is specific for D-glucose as exposure of the cells to 20 mM mannitol has no proliferative effect and leads to cell death by necrosis.

THE ACTIONS OF GLYCYRRHETINIC ACID DERIVATIVES ON HYPERPOLARIZATION OF IDENTIFIED ENDOTHELIAL CELLS IN ARTERIES

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Prominent endothelium-dependent hyperpolarization and relaxation persist in many arteries following inhibition of cyclooxygenase and nitric oxide synthase. This response is attributed to the actions of endothelium-derived hyperpolarizing factor (EDHF). The elusive nature of EDHF has fuelled speculation about its identity, with some studies indicating that it is a diffusible factor in some arteries while other reports suggest that EDHF-induced hyperpolarization in smooth muscle results from electrotonic spread of activity from endothelial cells via gap junctions. Glycyrrhetinic acid (GA) derivatives are triterpenoid saponins that inhibit intercellular transfer of metabolites and spread of fluorescent dyes between cells in various tissues and these actions have been attributed to the inhibition of gap junctions. Recently GA derivatives have been used to explore the involvement of gap junctions in the EDHF response, and they reduce the hyperpolarization attributed to EDHF in the smooth muscle. In this study we have investigated the actions of GA derivatives on dye-identified endothelial cells themselves.

Left main descending coronary arteries from guinea-pigs and mesenteric arteries from rats were removed from animals killed by cervical dislocation. For membrane potential recordings from endothelial cells, the arteries were cut open longitudinally and pinned to the floor of a recording chamber with endothelium uppermost. In other experiments ring segments of artery were mounted on a Mulvany-style myograph to enable simultaneous recordings of membrane potential and tension. Tissues were continuously superfused with warmed physiological saline. In all experiments N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) and indomethacin (1 μM) were included in the superfusate to inhibit nitric oxide synthase and cyclooxygenase, respectively.

In smooth muscle cells in rings of coronary artery mounted on the myograph, 18β-GA and another GA derivative, carbenoxolone, were both found to inhibit hyperpolarization attributed to EDHF. 18β-GA (30 μM) significantly reduced by 3 fold the hyperpolarization evoked by 1 minute exposure to 0.1 μM acetylcholine (ACh). In arteries depolarized and constricted with a thromboxane mimetic (U 46619), carbenoxolone (100 μM) also significantly reduced the EDHF hyperpolarization by 5 fold and accompanying relaxation by 3 fold. The actions of the GA derivatives were then studied in endothelial cells that were dye-identified, using Lucifer Yellow injection from the microelectrode. At rest, the mean amplitude of the hyperpolarization evoked by 1 minute exposure to 0.1 μM ACh in endothelial cells of the coronary artery was 17 ± 1 mV (n=10). Within 6 minutes 18β-GA had significantly reduced the ACh evoked hyperpolarization in the endothelial cells by 3 fold. Over a similar time frame, carbenoxolone also reduced the endothelial cell hyperpolarization by 4 fold. The EDHF-attributed hyperpolarization in endothelial cells of the rat mesenteric artery was also significantly reduced by 2 fold in the presence of carbenoxolone.

The significant finding from these experiments is that GA compounds inhibit the hyperpolarization attributed to EDHF recorded from dye-identified endothelial cells in guinea-pig coronary and rat mesenteric arteries. Similar results were also found in endothelial cells of guinea-pig submucosal arterioles. This limits the usefulness of GA compounds in the assessment of gap junction involvement in the EDHF response.

THE PROPAGATION OF MOTOR COMPLEXES IN THE ISOLATED MOUSE COLON

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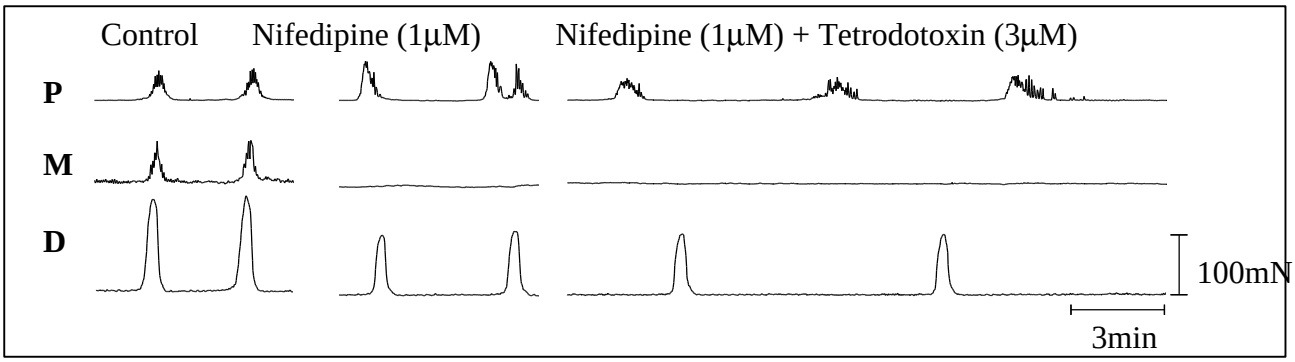
It has been proposed that the propagation of peristalsis in the intestine is dependent upon the sequential removal of an activated (by wall distension) descending inhibitory reflex by the contraction front. Spontaneous migrating contractions have been reported to occur in isolated preparations of mouse colon, without fluid distension (Fida *et al.*, 1997). Other experiments on this tissue have shown spontaneous propagating myoelectric complexes occur in the presence of a Ca^{2+}-channel antagonist (nifedipine, 1 μM) (Bywater *et al.*, 1989), which severely depresses contractile activity (Fida *et al.*, 1997). These results suggest that propagation of contractions in the isolated mouse colon may be dependent on mechanisms other than the removal of distension. The following experiments were designed to test whether contractile tone is required for the propagation of a **contraction**.

Mice were killed by CO_2 overdose followed by cervical dislocation. Colons were isolated and set up in a three-compartment organ bath, each separately perfused with carbogenated Krebs solution at 36°C. Circular muscle tension was measured separately in each compartment and the data digitised and stored on a PC. Spontaneous migrating contractions were recorded in 18 preparations, the majority showing oral-anal migration. The form of these contractions were similar to that reported by Fida *et al.*, (1997) using a single compartment organ bath, and occurred at intervals of 3.0 ± 0.19 min (n=18).

The addition of nifedipine (1 μM) to the middle compartment only, markedly depressed contractile amplitude in the mid colon, from 62.7 ± 13.8 to 2.4 ± 0.6 mN (P<0.01, n=6). Contractions continued to occur in the proximal and distal recording sites, without affecting their form or frequency. In the presence of nifedipine in the middle chamber, 5 of 6 preparations maintained their original migration direction. These results indicate that the contractions continued to migrate along the length of the colon, despite a >95% reduction of contractile amplitude in the middle compartment.

The addition of the neurotoxin tetrodotoxin (3 μM) to the middle compartment only still in the presence of nifedipine, abolished contractile activity in the mid colon, although resting tone transiently increased by ~10 mN, which decreased to a maintained tone increase of 3-5 mN (n=3). The association between contractions in the proximal and distal regions was disrupted, although contractions continued to occur in both regions. Contractions in the proximal region appeared less organised, with a change in the contribution of the phasic (relatively increased) and the tonic (relatively decreased) components of the contraction. Contractions in the distal region were of a similar form to those prior to the addition of tetrodotoxin, however with a tendency to slightly longer intervals, from 192 ± 31 to 329 ± 99s (n=3), however the difference was not significant (P=0.21).



These results suggest that migration of contractions along the mouse colon may not require generation of muscle tone for propagation but depend on neural connections between different sites.

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CHARACTERISATION OF THE SPONTANEOUS ACTION POTENTIALS RECORDED IN THE PROSTATE GLAND OF THE GUINEA-PIG

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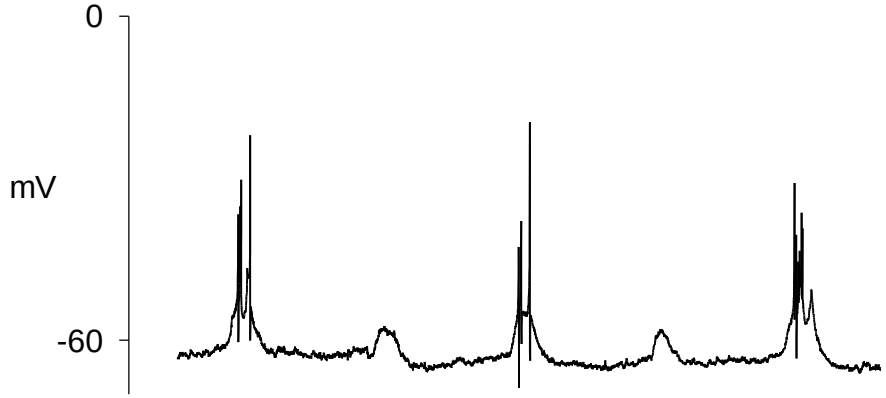
Contraction of the smooth muscle of the prostatic stroma, propels the prostatic secretions into the seminal fluid and transports them into the urethra during ejaculation. Although previous studies of the prostate gland have clearly established many of the basic contractile properties of this tissue, the intracellular mechanisms underlying the spontaneous activity of the prostate are essentially unknown. In this study, we present electrical recordings from the guinea-pig prostate gland. In addition, the effects of nifedipine, atropine and tetrodotoxin on the spontaneous action potentials are also examined.

The prostate gland was removed from guinea-pigs (250-350 g) killed humanely by stunning and exsanguination. Standard membrane potential recording techniques revealed at least 2 different cell types in the prostate gland. The first cell type was characterised by spontaneously-firing action potentials (see Figure) (*n*=34), presumably recorded from smooth muscle cells. The membrane potential, after-hyperpolarisation, frequency and duration of these cells was –53.6 ± 1.5 mV, -55.8 ± 2.1mV, 5.2 ± 0.4/s and 1.7 ± 0.2s, respectively.



The spontaneous electrical events appeared to be insensitive to blockers of neural propagation (1 μM tetrodotoxin, *n*=4) or cholinergic neurotransmission (1 μM atropine, *n*=2). In 75% of cells (6/8), the frequency of action potential discharge was little affected by the L-type calcium channel blocker, nifedipine at low (1 μM) (*n*=6) or higher concentrations (10 μM) (*n*=2). However, the amplitude of the mamimum spike was significantly reduced by 1 μM nifedipine from 44.6 ± 4.6 mV to 31.0 ± 4.6 mV ($P<0.05$), after 5 minutes. The remaining 2 spontaneously active cells were promptly abolished by nifedipine at the same concentration (1 μM). Electrical recordings were also frequently recorded from cells that were quiescent; action potential discharge could not be promoted in these cells using either a high K^+ (40-50 mM) saline or direct electrical stimulation (100 V, 5 s). The resting membrane potential of this cell type was –49 ±1.8mV (*n*=37). These cells may be epithelial in nature.

These results demonstrate that there are at least 2 types of cells recorded in the prostate gland of the guinea-pig, as revealed by electrophysiological techniques. Cells that were spontaneously active were mostly insensitive to nifedipine, tetrodotoxin and atropine, suggesting that the action potentials occurred myogenically and were somewhat independent of Ca^{2+} entry through L-type calcium channels.

EFFECTS OF PIROXICAM ON THE SLOW WAVES IN THE CIRCULAR MUSCLE OF THE GUINEA-PIG GASTRIC ANTRUM AND DUODENUM

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In circular smooth muscle cells of the guinea-pig gastric antrum, slow waves of large amplitude are driven by pacemaker cells (Interstitial cells of Cajal, Dickens *et al.*, 1999). In the guinea-pig small intestine, slow waves are readily recorded in vivo (Galligan *et al.*, 1985) but more rarely recorded in vitro (Smith 1989). It is possible that endogenous inhibitory substances are released from isolated dissected preparations. Prostanoids are synthesized in the intestine and inhibit circular muscle activity. Furthermore, prostaglandin synthesis inhibitors increase spontaneous circular muscle motor activity (Bennett *et al.*, 1976, Costa *et al.*, 1999), perhaps by removing an inhibitory "brake" mediated by endogenous prostanoids.

We have investigated the influence of piroxicam, a cyclooxygenase inhibitor, on myogenic slow waves in the circular muscle of the guinea pig antrum and duodenum in vitro, using intracellular recording. The gastric antrum, with 50 mm of duodenum, taken from guinea pigs killed humanely by cervical dislocation, was opened along the lesser curvature. Isolated preparations of antrum and duodenum were pinned flat, serosa uppermost, in a recording chamber. They were superfused with warmed oxygenated Krebs solution containing 2 µM nicardipine and 0.6 µM tetrodotoxin.

Slow waves were recorded from circular muscle cells in both regions. In the duodenum, but not in the antrum, they waxed and waned in amplitude. Duodenal slow waves exhibited a smooth wave-form with small amplitude, ranging from 1 to 5 mV. They had a frequency of 22.9 ± 0.2 cycles/min (n = 6, from 36 cells). After application of piroxicam (50 µM) for 45 minutes, the amplitude of duodenal slow waves was significantly increased from 2.0 ± 0.01 to 2.4 ± 0.1mV, but frequency was reduced to 20 ± 0.4 cycles/min ($P < 0.001$, n = 6). The duration of waxing periods of slow waves was also increased from 49.7 ± 2.9 to 74.9 ± 6.1 s ($P < 0.001$, n = 6), resulting in a significant reduction in the number of waxing periods from 4.8 to 3.3 per 5 min. Slow waves with a frequency of 4.6 ± 0.2 cycles/min and duration of 7.7 ± 0.2 s (n = 5, from 30 cells) were recorded in the antrum. They had a much larger amplitude, ranging from 17 to 36 mV, and exhibited four distinct components in the wave form. Piroxicam increased the amplitude and duration of antral slow waves from 25.5 ± 0.9 to 29 ± 0.8 mV ($P < 0.002$) and from 7.7 ± 0.2 to 8.4 ± 0.2 s ($P < 0.04$, n = 5) respectively. It also reduced the frequency of antral slow waves, slightly, but not significantly, to 4.4 ± 0.2 cycles/min.

These results suggest that the control mechanisms of myogenic activity in both the antrum and the duodenum are modulated by endogenous prostanoids

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ADRENERGIC BLOCKADE AND GLUCOSE KINETICS DURING STRENUOUS EXERCISE IN HUMANS

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The role of adrenaline in the regulation of glucose kinetics during strenuous exercise has not been fully elucidated. We recently demonstrated that while the intravenous infusion of adrenaline during low intensity exercise (40% VO_2 peak) to plasma levels attained during high intensity exercise (80% VO_2 peak) increased hepatic glucose production (HGP), it could not completely replicate the actual increase in HGP observed during exercise at the higher intensity (Howlett *et al.*, 1999). The present study was undertaken to further study the adrenergic regulation of glucose kinetics during strenuous exercise in humans by using combined α- and β-adrenergic blockade.

Six trained men (25.5 ± 4.9 yrs, 70.8 ± 6.6 kg, VO_2 peak = 4.18 ± 0.59 l min^{-1}, mean ± SD) reported to the laboratory in the morning after an overnight fast. Catheters were positioned in the radial artery for blood sampling and in a contralateral antecubital vein for tracer infusion. Subjects then ingested a capsule containing 5 mg prazosin hydrochloride and 5 mg timolol maleate (AB) and a primed, continuous infusion of 6,6 ^{2}H-glucose was commenced and maintained during a 2 hr rest period. Subjects then commenced supine cycling exercise for 20 min at a power output eliciting 81.9 ± 2.5% VO_2 peak (mean ± SE). Due to the earlier onset of fatigue in AB, the power output was reduced to 73.4 ± 2.9% VO_2 peak at 10 min and this was maintained until 20 min or the point of fatigue. Two subjects were unable to complete the full 20 min exercise bout, fatiguing at 16 and 19 min. On average, the exercise bout lasted 19.2 ± 0.7 min at 77.6 ± 2.7% VO_2 peak. Arterial blood samples were obtained at 5 min intervals during the last 15 min of the rest period and throughout exercise for subsequent measurement of plasma glucose, lactate and FFA concentrations and glucose tracer enrichment. Rates of glucose appearance (Ra = HGP in the fasted state) and disappearance (Rd) were calculated from the changes in plasma glucose concentration and glucose tracer enrichment using the non steady state Steele equations. At least one week later, subjects returned to the laboratory and repeated this protocol, but with pre-exercise ingestion of a placebo capsule (CON). There were no differences in oxygen uptake or RER during exercise between the two trials, but heart rate was lower ($P<0.05$) in AB (139 ± 8 beats min^{-1}) compared with CON (165 ± 5). Plasma glucose increased ($P<0.05$) in CON from 5.45 ± 0.10 mmol l^{-1} at rest to 6.48 ± 0.30 at the end of exercise. In contrast, plasma glucose did not change during exercise in AB (5.68 ± 0.30 vs. 5.68 ± 0.1). The blunting of the plasma glucose response in AB was due to a higher ($P<0.05$) glucose Rd, which averaged 30.6 ± 4.6 μmol kg^{-1} min^{-1} compared with 18.4 ± 2.5 in CON, since HGP during exercise was not different between trials (AB: 30.9 ± 4.4 vs. CON: 25.3 ± 3.9 μmol kg^{-1} min^{-1}). On average, plasma lactate was higher ($P<0.05$) in AB than CON (11.5 ± 0.8 vs. 9.4 ± 0.7 mmol l^{-1}), while plasma FFA were lower (0.12 ± 0.01 vs. 0.29 ± 0.04 mmol l^{-1}, $P<0.05$).

The results of the present study demonstrate that the blunted plasma glucose response to strenuous exercise with adrenergic blockade is due to enhanced peripheral glucose uptake, rather than reduced liver glucose output and suggest that adrenergic stimulation may be more important in "restraining" muscle glucose uptake than enhancing liver glucose production during strenuous exercise.

Howlett, K., Febbraio, M.A. & Hargreaves, M. (1999) American Journal of Physiology 276, E1130-E1135.

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THE EFFECT OF CARBOHYDRATE INGESTION ON METABOLISM DURING RUNNING AND CYCLING

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The effect of carbohydrate ingestion on metabolism was determined using conventional gas measurements combined with tracer-derived estimates of plasma glucose oxidation and direct measures of muscle glycogen disappearance. Seven moderately-trained males (mass 83.2 ±3.7 kg, peak oxygen uptake (VO_{2peak}) 54.1 ±1.5 and 51.1 ±1.9 ml kg min^{-1} for running and cycling, respectively) performed a random order of two running and two cycling trials lasting 60 min, at an intensity that corresponded to individual lactate threshold in that exercise mode. Training and food intake were controlled 24 h prior to each experimental trial: subjects refrained from vigorous exercise and consumed a standard diet of 6 g of carbohydrate kg^{-1} BM (body mass). Subjects ingested a total of 12 ml kg^{-1} BM of either a 6.4% carbohydrate-electrolyte solution (CHO) or water (W), with 8 ml kg^{-1} BM of fluid ingested before exercise and 2 ml kg^{-1} BM of fluid ingested after 20 and 40 min of exercise. As intended, there were no differences in blood lactate concentration between W and CHO for either running (2.0 ±0.1 vs. 1.7 ±0.1 $mmol^{-1}$ L) or cycling trials (2.1 ±0.3 vs. 2.1 ±0.2 $mmol^{-1}$ L). The area under the plasma glucose × time curve (AUC) was significantly greater during both running and cycling with CHO ingestion compared to W (Running: 405 ±13 vs. 363 ±13 Units; P <0.05 and Cycling: 380 ±10 vs. 334 ±7 Units; P <0.001). Similarly, the AUC (insulin × time) for running and cycling was significantly greater with CHO ingestion compared to W (Running: 454 ±52 vs. 322 ±14 Units; P <0.05 and Cycling: 571 ±121 vs. 332 ±46 Units; P <0.05). Although the respiratory exchange ratio (RER) was similar between treatments for running, the ingestion of CHO during cycling resulted in a significantly higher RER compared to W (0.92 ±0.01 vs. 0.90 ±0.01; P <0.05). Total carbohydrate oxidation was similar during both running (181 ±26 vs. 197 ±19 g h^{-1}) and cycling (157 ±25 vs. 162 ±15 g h^{-1}) trials for W and CHO treatments, respectively. A significant main treatment effect was found for plasma glucose oxidation during both running (65 ±20 vs. 42 ±16 g h^{-1}; P <0.01) and cycling (57 ±16 vs. 35 ±12 g h^{-1}; P <0.01), with CHO ingestion increasing the rate of plasma glucose oxidation compared to W. Accordingly, the contribution of plasma glucose oxidation to total carbohydrate oxidation was significantly greater with CHO than W ingestion during both running (33 ±4 vs. 23 ±3%; P <0.01) and cycling trials (36 ±5 vs. 22 ±3%; P <0.01). The ingestion of CHO when running or cycling did not result in a significant reduction in muscle glycogen utilisation compared to the W trial (P =0.595). However, there was a significant main treatment effect for exercise modality: muscle glycogen utilisation was significantly less during running than cycling when CHO was ingested (112 ±32 vs. 227 ±36 mmol glucosyl units kg^{-1} DM h^{-1}; P <0.01).

We conclude that the ingestion of CHO during running and cycling enhanced the contribution of plasma glucose oxidation to total carbohydrate oxidation compared to W. However, this increase did not result in a reduction in mixed muscle glycogen utilisation.

ADRENALINE INCREASES CARBOHYDRATE OXIDATION VIA ENHANCED SKELETAL MUSCLE GLYCOGENOLYSIS AND PDHa ACTIVITY

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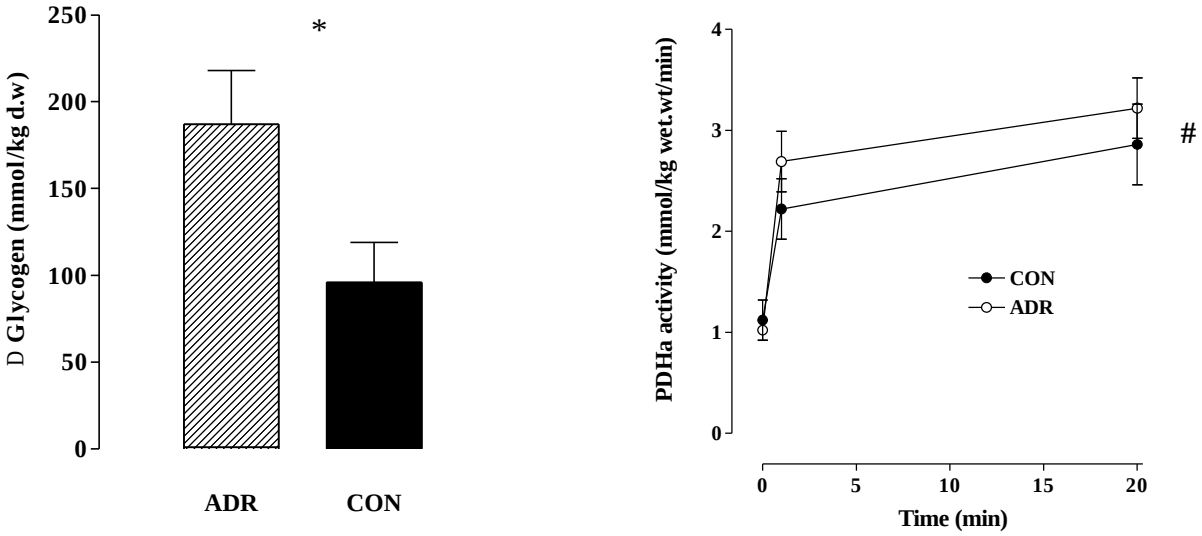
To investigate the effect of adrenaline on carbohydrate (CHO) metabolism during moderate exercise, we studied 10 active men (23.4 ± 3.2 years, 73.6 ± 14.2 kg, VO_2 peak = 4.2 ± 0.54 l min^{-1}, mean ± SD) during 20 min of cycle exercise at 58% VO_2 peak. On one occasion saline was infused (CON), and on the other exogenous adrenaline was infused intravenously for 5 min prior to and throughout exercise (ADR).

Infusion of adrenaline resulted in a progressive increase ($P<0.01$) in plasma adrenaline during exercise in ADR and values were three-fold higher at 20 min of exercise (1.41 ± 0.20 vs. 4.39 ± 0.91 nmol l^{-1} for CON and ADR, respectively, mean ± SE). In response to the higher plasma adrenaline, muscle glycogen breakdown increased two-fold (96 ± 23 vs. 187 ± 31 mmol kg^{-1} d.w for CON and ADR, respectively) and muscle lactate ($P<0.05$) and G-6-P ($P<0.055$, treatment effect) were similarly elevated. Muscle ATP, phosphocreatine, creatine and pyruvate concentrations were unaffected by adrenaline infusion. Plasma glucose, lactate and FFA were similar at rest, but were significantly elevated ($P<0.03$) in ADR compared with CON at 10 and 20 min of exercise. Glucose appearance was not different between trials; however, in response to adrenaline infusion, glucose disposal was decreased in ADR ($P<0.05$, treatment effect). Whole body carbohydrate oxidation, as measured by the respiratory exchange ratio, was 18% greater in ADR and could be fully accounted for by an increase ($P<0.01$, treatment effect) in PDHa activity (see Figure).



* denotes different from CON, $P < 0.05$; # denotes main effect for treatment, $P < 0.05$.

The data demonstrate that elevated plasma adrenaline levels during moderate exercise in untrained men can increase total carbohydrate oxidation. This increase in carbohydrate oxidation can be explained by greater skeletal muscle glycogen breakdown and enhanced PDHa activity. In addition, the decreased glucose uptake observed during exercise in ADR is likely to be the result of increased intracellular G-6-P and a subsequent decrease in glucose phosphorylation and metabolism.

SKELETAL MUSCLE nNOSm IN MALE ENDURANCE-TRAINED AND UNTRAINED HUMANS

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Given the role of nitric oxide in skeletal muscle glucose uptake (Bradley, Kingwell & McConell, 1999) and other skeletal muscle functions (Reid, 1998), much interest has focussed on whether skeletal muscle nitric oxide synthase (NOS) expression can be influenced by physical activity. Whereas nNOS protein was increased in rat skeletal muscle following treadmill exercise training (Balon & Nadler, 1997), 6 weeks of single-leg extensor training or running in humans failed to change skeletal muscle NOS protein or activity (Frandsen *et al.*, 2000). This study compared nNOSμ expression between male endurance trained (ET) and healthy untrained (UT) humans.

Vastus lateralis muscle biopsies were obtained from 10 ET cyclists and triathletes (Age: 25 ± 1 yrs, VO_2 max: 61 ± 1 ml kg^{-1} min^{-1} Values: Mean ± SEM) and 8 healthy UT individuals (Age: 26 ± 3 yrs, VO_2 max: 42 ± 1 ml kg^{-1} min^{-1}), and analysed for nNOSμ mRNA and protein. By RT-PCR, primers spanning exons 16-19 gave rise to a nNOS mRNA transcript in human skeletal muscle that confirmed the existence of nNOSμ, whilst sequencing confirmed the presence of the 102 nt insert between exons 16 and 17, identical to that previously reported (Lin *et al.*, 1998). Subsequent studies confirmed nNOSμ as the exclusive variant of nNOS expressed in human skeletal muscle. Solution hybridisation – RNase protection assay was used to determine nNOS-μ mRNA expression in 10 μg of total RNA extracted from 5 ET and 4 UT biopsy samples. Protected RNA hybrids were then analysed on non-denaturing polyacrylamide gel and quantitated by phosphorimaging. Western blot analysis was used to determine nNOS-μ protein expression. Following total protein extraction, skeletal muscle protein was purified using ADP-sepharose (which binds NADPH-binding proteins), and separated by SDS-polyacrylamide gel electrophoresis. Following transfer, nNOS-μ protein was detected using a specific human nNOS anti-body and luminescence detection onto film. The membrane was also probed for the endothelial isoform of NOS (eNOS).

There was no difference in nNOSμ mRNA expression between ET and UT (ET: 25 ± 2 vs UT: 23 ± 1 arb. units; $P > 0.1$). nNOSμ protein tended to be higher in ET compared with UT (1.87 ± 0.38 vs 1.00 ± 0.27 relative arb. units; $P = 0.097$). eNOS was not detected in any of the biopsy samples.

Unlike short term endurance training, which has been shown to have no effect on human skeletal muscle nNOS protein expression (Frandsen *et al.*, 2000), chronic endurance physical activity may be associated with an increase in skeletal muscle nNOS protein expression. If confirmed, such a finding may have important implications for the regulation of skeletal muscle function.

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EFFECT OF FASTING ON GENE EXPRESSION IN HUMAN SKELETAL MUSCLE

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Fasting triggers a complex array of adaptive metabolic and hormonal responses including an augmentation in the capacity for mitochondrial fatty acid oxidation in tissues such as skeletal muscle. The processes by which adaptations occur in response to variations in metabolic substrate availability require changes in gene expression causing a modification in the concentration of specific proteins critical for the function of a metabolic pathway. This study examined the effect of fasting on the transcriptional regulation of genes important for metabolic control in human skeletal muscle. Seven healthy subjects (4 male, 3 female, age 24.7 ± 4.8 years, weight 63.3 ± 10.6 kg, BMI 21.9 ± 1.6 kg m^{-2}, mean ± SD) undertook a 40 hour fast with resting biopsies and blood samples taken 3, 16, and 40 hours following consumption of a standard meal (25% fat, 60% CHO, 15% protein). Gene expression of carbohydrate metabolism enzymes pyruvate dehydrogenase kinase (PDK) isoforms 1,2,3 and 4, and lipid metabolism proteins fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (mASPAT/FABPpm), carnitine palmityl transferase I (CPT I), β-hydroxyacylCoA dehydrogenase (β-HAD), acyl coenzyme A: diacylglyceral acyltransferase (DGAT) and hormone sensitive lipase (HSL) were analysed using Taqman PCR. Following 40 hours fasting, pyruvate dehydrogenase (PDH) activity decreased 60% ($P<0.05$), while PDK 4 gene expression increased 12 fold ($p<0.05$). The other abundant isoform, PDK 2, did not change, whilst isoforms 1 and 3 were expressed at very low levels and did not respond to fasting. There were no significant effects of fasting on gene expression of key proteins involved in lipid metabolism. These data demonstrate that in human skeletal muscle, PDH activity is dramatically reduced, accompanied by increased gene expression of the inhibitory PDK isoform 4. On the other hand, the gene expression of key proteins involved in the transport and oxidation of lipids was not significantly altered. This is suggestive of regulatory control of fat oxidation in response to fasting, independent of gene expression.

METABOLIC RESPONSES TO INTENSE EXERCISE IN WELL-TRAINED HUMANS

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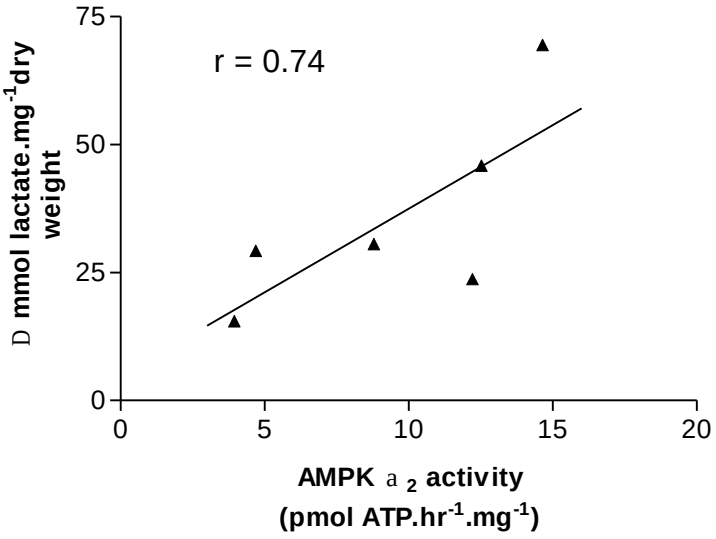
To determine the metabolic demands associated with intense exercise, seven well-trained cyclists (maximal O_2 uptake [VO_{2max}] 5.14 ±0.23 L min^{-1}, peak sustained power output [PPO] 405 ±22 W, values are mean ±SD), who were riding 370 ±140 km wk^{-1}, performed 8 × 5 min work bouts at 86 ±2% of $VO_{2\ max}$ (334 ±18 W) with 60 s recovery. Muscle biopsies were obtained from the vastus lateralis before and immediately after the training session, while pulmonary gas exchange and blood were sampled at regular intervals throughout exercise. Muscle glycogen content decreased from 501 ±91 to 243 ±51 mmol kg^{-1} dry mass (d.m.) (P<0.01), while muscle lactate concentration increased from 6 ±1 at rest to 32 ±12 mmol kg^{-1} d.m. at the completion of the training session (P<0.01). High rates of carbohydrate oxidation were maintained throughout exercise (~340 μmol kg^{-1} min^{-1}), while rates of fat oxidation increased from 16 ±8 during the first, to 25 ±13 μmol kg^{-1} min^{-1} during the seventh work bout (P<0.05). Blood lactate concentration was elevated to between 5-6 mmol l^{-1}, while blood pH remained at ~7.3 throughout exercise. Arterial oxygen saturation (%SpO_2a) decreased to 96 ±1% after the first work bout, falling to 94 ±2% after bout seven. The 60 s rest intervals were adequate to restore %SpO_2a to ~97%. Adenosine 5'-monophosphate-activated protein kinase (AMPK) α_1 activity was not altered by intense exercise (~ 7.1 ± 2.5 pmol adenosine triphosphate [ATP] h^{-1} mg^{-1}). However, AMPKα_2 activity increased 111% (from 4.2 ±1.7 to 9.5 ±4.4 pmol ATP h^{-1} mg^{-1}) in response to intense cycling (P < 0.05). The post exercise AMPKα_2 activity was significantly correlated to the change in muscle lactate concentration over the exercise session (r = 0.74, P < 0.05) as shown in the Figure below.



In conclusion these data show that well-trained cyclists can sustain high steady-state aerobic power outputs that are associated with high rates of glycogenolysis and total energy expenditure. Such metabolic demands result in an isoform specific activation of AMPKα_2.

EFFECT OF MODE OF EXERCISE AND CHO INGESTION ON IL-6 GENE EXPRESSION IN HUMAN SKELETAL MUSCLE

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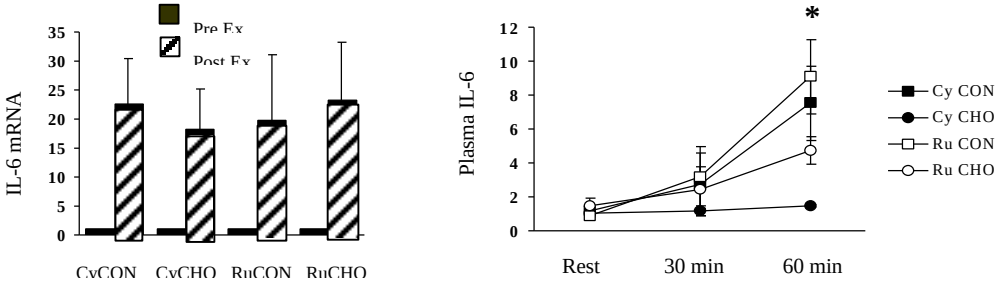
Exercise increases plasma concentrations of the pro-inflammatory cytokine interleukin (IL)-6. It has been demonstrated that this increase is augmented when comparing running with cycling exercise and blunted by carbohydrate (CHO) ingestion, irrespective of exercise mode (Nieman *et al.*, 1998). Although it has been hypothesised that monocytes are the source of the rise in plasma IL-6, we have demonstrated that neither exercise nor CHO ingestion affects monocyte intracellular IL-6 production (Starkie *et al.*, 2000). In addition, IL-6 mRNA is elevated in skeletal muscle after exercise, suggesting that contracting muscle is a probable source of the exercise induced increase in IL-6 (Ostrowski *et al.*, 1998). Hence, the aim of the present study was to examine the effect of mode of exercise and CHO ingestion on IL-6 gene expression in human skeletal muscle.

Seven moderately trained men (31 ± 2 yrs, 83.2 ± 3.7 kg, VO_2 peak = 4.39 ± 0.23 l min^{-1}, mean ± SD) completed four randomised exercise trials for one hour at a workload corresponding to each individuals lactate threshold. Two trials were conducted on a bicycle ergometer (Cy) and two on a running treadmill (Ru) either with (CHO) or without (CON) the ingestion of a CHO beverage throughout the exercise. Muscle biopsies were obtained before and immediately after exercise in all trials and these samples were measured for IL-6 gene expression using real time PCR. In addition, blood samples were collected at rest, 30 min during and at the cessation of exercise, which were analysed for plasma IL-6. Exercise resulted in a 21 ± 4 fold increase in IL-6 mRNA expression. However, this increase was not affected by either mode of exercise or CHO ingestion (see Figure). In contrast, whilst mode did not influence plasma IL-6 concentration, CHO ingestion blunted the plasma cytokine response (P<0.01 for beverage x time interaction) (see Figure).



These data demonstrate that CHO ingestion attenuates plasma IL-6 concentration during both cycling and running exercise. However, since IL-6 gene expression was unaffected by CHO ingestion it is probable that the ingestion of CHO attenuates IL-6 production by other tissues.

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DOES END TIDAL PCO_2 AT THE END OF EXERCISE PREDICT SEVERITY OF EXERCISE INDUCED ASTHMA IN CHILDREN?

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Exercise induced asthma (EIA) is characterised by airway narrowing which occurs typically post exercise. Recently Baba *et al.* (1997) have suggested that in some children with EIA, bronchoconstriction leads to a relative hypoventilation during exercise and is characterised by elevated levels of $P_{ET}CO_2$ and reduced ventilatory equivalent for CO_2. We have examined responses to exercise in a large group of asthmatic children to determine whether severity of EIA as indicated by post exercise airway narrowing can be predicted by ventilatory responses near the end of exercise.

78 children (30 girls, aged 6.9-11.9 yrs) with doctor diagnosed asthma and no preventive pre-medication, completed an 8 minute incremental treadmill walk/run to 60% MVV, while breathing dry air (Anderson, 1993). Metabolic parameters were monitored throughout exercise with a Jaeger Oxycon Record and were averaged and displayed in real time every 10s. EIA was assessed by the % Fall Index in FEV_1 in the 15 minutes post exercise, with a fall of >13% being taken as a positive response (Godfrey *et al.*, 1999). A comparison between mean results (±SEM) of EIA and non EIA groups is shown in the table.

Parameter	Non EIA	EIA	Significance
n	35	43	ns
age	9.75 ± 0.20	9.70 ± 0.24	ns
boys/girls	24/11	24/19	ns
%fall index in FEV_1	6.5 ± 0.08	34.8 ± 2.3	sig
FEV_1 % predicted	94.9 ± 2.1	83.2 ± 2.1	sig
$P_{ET}CO_2$ (torr)	34.8 ± 0.4	34.4 ± 0.5	ns
V_E/VCO_2	31.8 ± 0.6	32.9 ± 0.5	ns
VO_2 (ml kg^{-1} min^{-1})	41.7 ± 1.4	39.5 ± 1.0	ns
%target V_E	98.0 ± 2.2	98.8 ± 1.4	ns
%pred maxHR	88 ± 1.1	89 ± 1.0	ns
RER	0.96 ± 0.009	0.96 ± 0.009	ns

While $P_{ET}CO_2$ and V_E/VCO_2 were closely correlated ($P<0.001$) there was no significant correlation with % fall index in FEV_1 and either $P_{ET}CO_2$ or V_E/VCO_2 at the end of exercise. It is unlikely therefore that monitoring $P_{ET}CO_2$ during exercise is a reliable indicator of severity of EIA in asthmatic children.

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DEMONSTRATION OF SIMULATION, NAVIGATION AND QUESTIONS AS TUTORIAL INTERACTIONS

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We have previously discussed the structure of interactions used in CAL tutorials, in particular the construct of items which cascade from open to closed questions (Huxham, 1994). We now wish to extend our discussion to include other forms of interaction and to reflect on the educational value of these.

We have used Macromedia Authorware to create CAL tutorial interactions, simulations were written using Authorware's scripting language, animations have been written using Macromedia Director, static graphics using Aldus SuperPaint and Adobe Photoshop, and video material digitised using both QuickTime and Microsoft Media Player

This demonstration will illustrate the use of interactions involving cascading from open to closed questions, navigation used as a learning tool and simulations with embedded problems. Cascading interactions will be demonstrated using the CAL's 'Managing an Infarct' and' Cardiovascular Mechanics', navigation is used as a learning tool in the CAL 'Chest X ray' and our use of simulations will be shown in 'Control System' and 'Cardiac vectors'.

When computer assisted learning programs were first introduced in 1991 they were perceived by the students as innovative and exciting and evaluations carried out at the time gave them a higher rating than other teaching resources (Huxham *et al.*, 1992). Since then increased use of such material has meant that in order to appeal to students new strategies must be introduced (Oelrichs, 2000). We have found simulations like Human and MacPuff etc have been well received by the students. However the complexity of the interactions and the difficulty in determining starting values means that close supervision and extensive written instructions are required to encourage the active use of the tutorial and to facilitate reflection and insight. To facilitate use by the students we provided simple simulations. These were not widely used until the tutorial and self test components were combined and introduced with motivating information indicating significance; becoming a 'stand alone' tutorial.

We have found that, with cascading open to closed items, and despite claims to the contrary, students frequently do not take the opportunity to reword the answers to open questions. They re-enter the same answer until the opportunity to respond to the alternative, a multiple choice item, is provided. Inclusion of a navigational structure for the examination of a chest radiograph drills the student in using a systematic approach while, at the same time, enabling learning about the physiology and mechanical properties of the lung. This is particularly useful in a course where the discipline of physiology is integrated with other disciplines and the motivation is to develop skills revenant to medical diagnosis. Three-dimensional graphics and videotaped experiments encourage use of the material and add to its appeal but not necessarily to its educational value. Electronic tutorials must be attractive, accessible and relevant to compete with increasing available resources from the World Wide Web.

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EFFECTS OF EXOGENOUS ADENOSINE ON SPHINCTER OF ODDI AND DUODENAL CONTRACTILE ACTIVITY IN VITRO

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The biliary system develops from a diverticulum of the small intestine and therefore ganglia found within the biliary tree are usually considered part of the enteric nervous system. Purinergic substances are known modulators of gastrointesintal motility, however few studies have investigated the role of purines on biliary motility. The aim of this study was to determine if exogenous adenosine affects the motor activity of the sphincter of Oddi (SO) in a similar manner to its effect on duodenal motor activity.

The SO and duodenum were removed from fasted Australian Brush-tailed possums (*Trichosurus vulpecula*) after induction of anaesthesia by intramuscular injection of ketamine (20 mg/kg) and xylazine (5 mg/kg). The animals were then euthanased with a lethal dose of sodium pentobarbitone. The SO was dissected into 4 equal, 'figure-8' muscle rings and allocated to either a distal SO (*juxta* duodenum) or proximal SO group. Longitudinal duodenal muscle strips were prepared. Each muscle ring/strip was secured in an organ bath, muscle contractility was measured isometrically and recorded using a MacLab recording system. After a 30 minute equilibration period, stable spontaneous activity was recorded. Single concentrations of adenosine (0.5×10^{-4} to 10^{-3}M) were randomly added to SO muscle rings (n=4 for each SO group). For duodenal strips, adenosine (10^{-6} to 10^{-3}M) was added non-cumulatively for 5 minutes per concentration (n=5). To determine if the response was neurally mediated, adenosine was re-applied following pre-treatment with tetrodotoxin (TTX, 10^{-6}M). Recordings of muscle activity were analysed for contraction frequency (contractions/min), contraction amplitude (mN), basal tone (mN) and area under the contraction (AUC; mN.s) for 1 min before the addition of adenosine (control) and during a 1 min period representing the maximum response following adenosine application. Statistical analysis utilised Mann-Whitney and Kruskal-Wallis analyses, univariate analysis of variance or repeated measures analysis of variance, as appropriate.

Inhibitory responses to adenosine were observed with SO muscle rings. The distal and proximal SO groups responded differently with regard to AUC (P<0.01) and contraction amplitude (P<0.04). Further analysis identified a concentration-dependent response for the proximal SO group only. Contraction frequency was decreased (P<0.01) but was not different between SO groups. In comparison adenosine induced a bi-phasic response in duodenal muscle strips. The response consisted of a very rapid and short-lasting increase in basal tension (P<0.001) followed by a long-lasting, concentration-dependent decrease in basal tension (P<0.001) and AUC (P<0.001). Pre-treatment with TTX had no apparent effect on the adenosine-induced response for so rings or duodenal strips.

In summary, exogenous adenosine has an inhibitory effect on the SO, but acts differently at the distal and proximal sites. Duodenal tissue exhibited a bi-phasic response to adenosine. The insensitivity of the response to TTX suggests that adenosine mediates its response via receptors on the smooth muscle. These findings indicate that adenosine is a modulator of motor activity in the biliary tree and duodenum, however different receptor populations appear to exist on the SO compared to the duodenum.

INFLUENCE OF CENTRAL ANGIOTENSIN II IN THE RENAL NERVE INHIBITION ELICITED BY VOLUME EXPANSION IN THE CONSCIOUS RABBIT

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Central angiotensin II plays an important role in the regulation of blood pressure and blood volume. ANGII binding sites are found in the nucleus tractus solitarius and in the ventrolateral medulla which are brain regions crucial to the normal regulation of sympathetic nerve activity (SNA). Central ANGII may contribute to the elevated SNA characterising chronic fluid overload conditions such as congestive heart failure (CHF). Volume expansion elicits a marked reflex reduction in SNA and this reflex is attenuated in CHF, which may contribute to the abnormal SNA in CHF. The role of central ANGII in the reflex is unknown. Thus, the aim of the present study was to determine whether endogenous ANGII within the brain participated in the reflex renal nerve inhibition elicited by VE in the normal conscious rabbit.

At least two weeks before experiments, NZW male rabbits (*n*=9) were prepared with renal nerve electrodes and a brain ventricular (IV) catheter in separate surgical procedures using ketamine/xylazine (40 mg/kg i.m. / 5 mg/kg i.m.) general anaesthesia. There were two experimental days; on one day conscious rabbits were administered an intraventricular injection of losartan (10 μg) that was sufficient to block the pressor response induced by central ANGII (50 ng). Then the rabbits were volume expanded with the plasma expander, Haemaccel (2 ml/min, i.v., 30 minutes). On the other day (control), Ringers replaced losartan. Reflex RSNA, mean arterial pressure (MAP) and heart rate (HR) were monitored and compared between treatments using a two-way ANOVA with repeated measures.

We found that in the conscious rabbit losartan or Ringers administered into the fourth ventricle did not affect basal MAP, HR nor RSNA. Following Ringers administration into the fourth ventricle volume expansion elicited a significant reflex reduction in RSNA of approximately 45%. Following losartan pretreatment, volume expansion reflexes reduced RSNA by 33%. There was no significant difference between the two treatments. MAP and HR during the volume expansion were also not different between the treatments.

We conclude that endogenous ANGII is not involved in the tonic maintenance of RSNA, MAP nor HR in the conscious rabbit. Central endogenous ANGII does not appear to make a major contribution to the renal sympatho-inhibitory response elicited following acute volume expansion in the normal conscious rabbit.

THE ROLE OF NICOTINIC AND P2X RECEPTORS IN DESCENDING EXCITATION IN THE GUINEA-PIG ILEUM

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Electrophysiological studies of the guinea-pig ileum have demonstrated that physiological stimuli applied to the intestinal wall evoke excitation in the circular muscle oral to the stimulus and inhibition anal to the stimulus; ascending excitation and descending inhibition, respectively. The descending inhibition does not depend on nicotinic transmission in the reflex pathways, but is depressed by blockade of neuronal P2X receptors (Bian *et al.*, 2000). In recent studies in the guinea-pig ileum, the contractile responses to either distension or mucosal distortion have been found to lead to an anally propagating contraction (descending excitation) that is only slightly modified by descending inhibition (Spencer *et al.*, 1999). Pharmacological studies of the pathway mediating the descending excitation, however, have yielded ambiguous results (Spencer *et al.*, 2000). The present study reinvestigated the role of nicotinic and P2X receptors in descending excitation to determine how this pathway relates to that responsible for descending inhibition.

Guinea-pigs were killed by being stunned and then having their carotid arteries and spinal cords severed. Segments of ileum (~7 cm in length) were then dissected from the animal, opened along their mesenteric borders and pinned out mucosal side up in an organ bath where they were superfused with physiological saline at 36^{o}C and bubbled with 95% O_2, 5% CO_2. Distensions were applied to the oral end of the preparation by inflating a balloon set into the base of the organ bath and contractile responses recorded from both the longitudinal and circular muscle layers at the anal end with isotonic transducers.

Distension elicited separate contractions in both the circular and longitudinal muscle layers with no preceding relaxation in either case. These responses were abolished by the application of tetrodotoxin (1 μM) to the bath, while the addition of hexamethonium (500 μM) had no significant effect on either muscle layer. The addition of the P2 receptor antagonist pyridoxal phosphate-6-azopheyl-2',4'-disulphonic acid (PPADS; 10 μM) to the bath decreased both the circular and longitudinal muscle responses to 36 ± 11% (mean ± standard error of mean) and 42 ± 10% of the control response, respectively. Hexamethonium (500 μM) and PPADS (10 μM) added together reduced the circular muscle response to 16 ± 8% of control. Preliminary experiments using the 5-HT_3 receptor antagonist granisetron (1 μM) indicate that these serotonin receptors may also play some role in the descending excitatory pathway.

These results confirm that the descending excitation is neurally mediated. They also indicate that there are substantial similarities between the descending excitatory and descending inhibitory pathways. In both pathways, acetylcholine acting at nicotinic receptors does not appear to have a major role in the transmission of reflex activity, while ATP at P2X purinoceptors appears to have a substantial role. This is somewhat surprising as activity in the ascending excitatory pathway is critically dependent on nicotinic transmission.

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REGRESSION OF CARDIAC HYPERTROPHY IN THE SHR BY RENIN-ANGIOTENSIN SYSTEM BLOCKADE AND DIETARY SODIUM RESTRICTION

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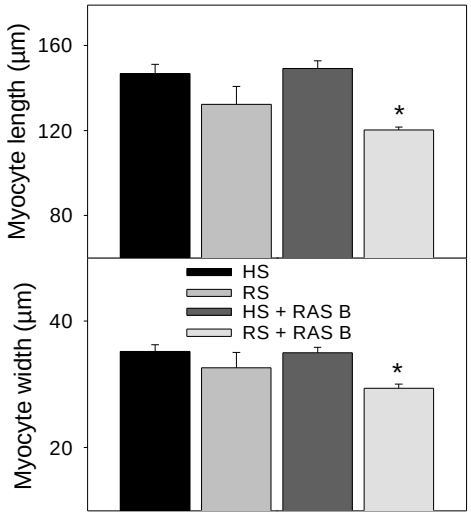

***P<0.01 vs HS + RAS-B**

Combined angiotensin converting enzyme inhibition (ACEI) and angiotensin type-1 receptor (AT_1) blockade offers new potential in the treatment of hypertension and cardiac hypertrophy. We previously demonstrated that combination treatment with losartan and perindopril caused pronounced hypotension and cardiac atrophy in normotensive Sprague-Dawley rats (Griffiths *et al.*, 1999). These effects were prevented in animals receiving a high sodium chloride (NaCl) diet suggesting that NaCl intake may be important during such treatment. In the present study we examined the effects of varied dietary NaCl intake and combined treatment with an ACEI together with the insurmountable AT_1 blocker candesartan in spontaneously hypertensive rats (SHR) with established cardiac hypertrophy.

Adult male SHR (16-20 weeks) were fed high (4%) or reduced (0.2%) NaCl chow for 14 days. On days 7-14, i.p. injections of candesartan (3 mg/kg) and perindopril (6 mg/kg) were given (RAS-B). Diet only treated animals received vehicle injections. At day 14 tail-cuff blood pressures and body weights were measured. Animals were anaesthetized (pentobarbitone sodium, 60 mg/kg, i.p.) and hearts removed and weighed. Cardiac myocytes were isolated enzymatically and their dimensions were measured by widefield microscopy.

Treatment	Weight gain (g)	Heart weight (mg)	Cardiac Index (mg/g)
High NaCl	22 ± 5.1	1470 ± 15.2	3.88 ± 0.03
Reduced NaCl	34.2 ± 3.1	1452 ± 29.9	3.74 ± 0.08
High NaCl + RAS-B	26.6 ± 3.2	1372.6 ± 21.5	3.72 ± 0.10
Reduced NaCl + RAS-B	-21.6 ± 6.4*	1023.4 ± 40.2*	3.22 ± 0.04*

**P*<0.05 vs all other groups (*n*=5 animals each).

The Table shows the effect of the four treatments on body weight and cardiovascular parameters. Heart weight and cardiac index was decreased in the reduced NaCl + RAS-B group compared to all other groups, and these animals lost body weight (Table). A comparison of the myocyte dimensions among the treatment groups (Fig.) shows that cardiac myocytes from the reduced NaCl + RAS-B group were smaller in length and width, but that myocyte size was not affected in animals receiving high NaCl + RAS-B. The systolic blood pressure difference between these groups was significantly different (high NaCl + RAS-B, 161 ± 9; reduced NaCl + RAS-B, 100 ± 8; *P*<0.01).

These results indicate that combined RAS blockade with candesartan and perindopril is highly effective at regressing cardiac hypertrophy in hypertensive SHR on a reduced NaCl intake. The regression of hypertrophy reflects a reduction in cardiomyocyte dimensions. Hypertrophic regression was not observed when RAS blocker treatment was given in association with a high NaCl intake.

Griffiths, C.D., Delbridge, L.M.D. & Morgan, T.O. (1999) Proceedings of the Australian Physiological and Pharmacological Society, 30(2), 76P.

Supported by the NH&MRC of Australia. Candesartan generously donated by Astra-Zeneca, Sweden.

U46619-INDUCED CA^{2+} TRANSIENTS IN GUINEA-PIG MESENTERIC LYMPHATIC SMOOTH MUSCLE

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It is well known that an increase of intracellular calcium concentration ($[Ca^{2+}]_i$) play an important role in triggering constriction of vascular smooth muscle through an influx of Ca^{2+} across the sarcolemma and/or release of Ca^{2+} from intracellular stores. Studies on guinea-pig mesenteric lymphatics have shown that lymphatic vasomotion, a rhythmic dilation and constriction cycle of lymphatic vessels occurs through Ca^{2+}-mediated action potentials triggered by a summation of spontaneous transient depolarizations (STDs, Van Helden, 1993), generated by synchronised 'quantal' release of Ca^{2+} from inositol 1,4,5-trisphosphate ($InsP_3$) receptor-operated intracellular stores. (Van Helden, 1993; Van Helden *et al.*, 1996).

It was shown previously that U46619, a stable analogue of thromboxane/endoperoxide increases lymphatic vasomotion by directly acting on the lymphatic smooth muscle (Rayner & Van Helden, 1997) and induces/enhances spontaneous transient depolarizations (STDs) and resultant action potential-mediated Ca^{2+} transients and associated vasomotion (von der Weid *et al.*, 1999). The present experiments investigated U46619-associated mechanisms underlying lymphatic vasomotion.

Experiments were performed on mesenteric lymphatics freshly isolated from young guinea-pigs killed by cervical dislocation during deep anaesthesia consequent to inhalation of halothane. The mesentery was pinned in a bath and superfused with physiological saline. The endothelium of lymphatics was removed by repeatedly (5-6 times) passing brief (5-10s) streams of air through the lumen of the vessels in which a fine glass cannula was loosely inserted. $[Ca^{2+}]_i$ in the smooth muscle was measured ratio metrically using the calcium sensing dye fura 2/AM (Molecular probes) by a photometer based system. The smooth muscle was loaded at 35°C by 30 min perfusion of endothelium lysed vessels with 2 μM fura 2/AM added to the luminal perfusate followed by a 5 min washout. Preparations were mounted onto a small metal ring, placed in a glass-bottomed organ bath (volume 0.5 ml) and viewed with an inverted microscope in a dark room. Tissues were superfused with physiological saline heated to 34-36°C at a rate of 5 ml/min. Vessel segments were exposed to UV excitation for 100 ms every second being exposed to 340 nm and then 380 nm from a Xenon light for 50 ms each with output light passed through a dichroic mirror of 490 nm and a bandpass filter of 510 nm. Ratiometric images were collected every second with results recorded by computer.

Maintained application of U46619 (10^{-7} M) induced responses that exhibited some tachyphylaxis but generally Ca^{2+} transients lasted for >30 min and were constant in amplitude and frequency. SQ-29548 (3×10^{-7} M), a selective thromboxane A_2 receptor antagonist, nifedipine (10^{-6} M), a L-type Ca^{2+} channel blocker, CPA, a store ATPase inhibitor, caffeine (10 mM) and removal of extracellular Ca^{2+} together with 1mM EGTA abolished the U46619-induced $[Ca^{2+}]_i$ transients, whereas ryanodine (20 μM) had no significant effects on the U46619-induced responses. The results indicate that U46619 triggers persistent action potential-related Ca^{2+} transients in guinea-pig lymphatic smooth muscle by activating thromboxane receptors. The data are consistent with U46619 enhancing pacemaking by increasing Ca^{2+} release from inositol 1,4,5-trisphosphate receptor-operated Ca^{2+} stores and that action potentials and resultant Ca^{2+} transients generated by the underlying pacemaker activity are mediated by L-Ca^{2+} channels.

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EFFECT OF cSrc INHIBITION ON MYOGENIC RESPONSES AND PHOSPHOTYROSINE FORMATION IN ARTERIOLES

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The mechanisms underlying myogenic constriction of arterioles in response to increased transmural pressure are not fully understood. Our previous studies showed an increase in protein tyrosine phosphorylation within the smooth muscle cells of a cannulated arteriole *in vitro* following an increase in transmural pressure (Murphy *et al.*, 1998), however the tyrosine kinases involved were not identified. One possible candidate is the cSrc-family of protein tyrosine kinases. The present study examined the effect of the selective cSrc-type protein tyrosine kinase inhibitor PP1 on pressure-induced contraction and phosphotyrosine formation in arterioles from rat skeletal muscle.

First–order arterioles were dissected from the excised rat cremaster muscle of anaesthetised (sodium thiopentone 100 mg/kg, i.p.) Sprague-Dawley rats which were then killed. The arterioles were cannulated on micro-pipettes and mounted in a superfusion chamber as described previously (Murphy *et al.*, 1998). Vessels were pressurized to 70 mmHg and the passive diameter of the vessels was taken as the maximum dilation to 10 μM acetylcholine. Following this procedure the intra-luminal pressure was reduced to 30 mmHg and the vessel was incubated with vehicle or PP1 (10 μM) for 20 min. At the conclusion of this period the intra-luminal pressure was increased to 100 mmHg and maintained for 15 or 60 min, in the continued presence of vehicle or PP1. Eight additional arterioles were maintained at 70 mmHg and exposed to angiotensin II (AII) for 5 min, in the presence or absence of PP1 (10 μM). At the conclusion of the treatment period all vessels were fixed and examined for total phosphotyrosine content using FITC-conjugated anti-phosphotyrosine and confocal microscopy as described previously (Murphy *et al.*, 1998).

In control experiments the diameter of vessels maintained at 30 mmHg was 53.2 ± 4.5% of passive diameter (n=4). Following an increase in transmural pressure to 100 mmHg, vessels underwent a passive distention followed by myogenic constriction. Arteriolar diameter was 44.0 ± 4.7% of passive after 15 min and 49.5 ± 6.5% of passive (n=4) after 60 min at 100 mHg. In the presence of PP1 myogenic constriction was not altered significantly at 15 min (44.7 ± 3.7%) but arterioles were significantly dilated after 60 min (65.9 ± 5.6%, P<0.05, n=4 for both). In measurements of phosphotyrosine formation, PP1 did not reduce anti-phosphotyrosine fluorescence after 15 min at 100 mmHg (control, fluorescence intensity = 32.6 ± 1.1 units; PP1, 35.4 ± 3.5 units) or 60 min (control, 40.3 ± 3.7 units; PP1, 40.3 ± 2.6 units). AII (0.1 μM) constricted pressurised (70 mmHg) arterioles, PP1 did not alter AII-induced constriction (20.9 ± 2.8 % of passive, n=4) but did reduce AII-induced phosphotyrosine formation from 61.8 ± 4.8 units to 36.7 ± 5.8 units (P<0.05).

This study showed an effect of PP1 on the maintained arteriolar constriction following an acute increase in transmural pressure. In contrast, during the early phase of myogenic constriction (0-15 min), PP1 had no effect, suggesting a role for cSrc in maintained myogenic tone as opposed to acute myogenic constriction. However PP1 did not reduce tyrosine phosphorylation, suggesting the contribution of cSrc-phosphorylated species to the pressure-induced increase in total phosphotyrosine may be minor. PP1 reduced phosphotyrosine formation in response to AII, but did not alter constriction, suggesting no role for cSrc in AII-induced vascular smooth muscle contraction but possibly other responses such as cell growth.

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COMPARISON OF TWO ORTHOSTATIC CHALLENGES – THE +75^0 HEAD-UP TILT AND THE SQUAT-STAND TEST

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Head-up tilt (HUT) has been widely used in both research and clinical settings as an orthostatic challenge for investigating cardiovascular reflexes. However in recent years the squat-stand test (SST) has been introduced as an alternative to HUT. There is much contention as to whether these two orthostatic tests produce equivalent cardiovascular challenge. This study was undertaken to compare these two methods of orthostatic challenge.

Five females (21.8 ± 1.9 yrs; 1.66 ± 0.09 m; 60.7 ± 7.5 kg) and five males (25.6 ± 4.2 yrs; 1.80 ± 0.04 m; 76.4 ± 7.5 kg) underwent +75^0 head-up tilt (HUT) and a squat-stand test (SST). Mean arterial pressure (MAP) and heart rate (HR) were determined non-invasively using a Portapres^{TM} Model 2.0. Data was recorded on a beat-to-beat basis 10 sec prior to (control) and 10 sec after tilt or stand (event) for the HUT and SST respectively. Event MAP and HR responses were compared and analysed by calculating the deviation from control MAP and control HR for both tests. A test for statistical agreement was performed according to the method described by Bland and Altman (1986).

The MAP and HR responses were significantly different between the two tests, with responses to the SST involving a greater reduction in MAP and a greater increase in HR. The average MAP responses (deviation from control) for HUT and SST were –9.0 mmHg (± 8.3 mmHg) and –21.6 mmHg (± 8.6 mmHg) respectively (P<0.05). The average HR responses (deviation from control) for HUT and SST were 15 bpm (± 7 bpm) and 22 bpm (± 7 bpm) respectively (P<0.05). There was poor statistical correlation (r=0.13) and poor statistical agreement between the MAP responses for the two orthostatic challenges. Conversely there was good statistical correlation (r=0.76) and good statistical agreement between the HR responses for the HUT and SST.

Based on the MAP response, the results of this study suggest that HUT and SST are two physiologically different orthostatic challenges. It appears that the active nature of the SST leads to a more pronounced change in MAP compared to the passive action of HUT. As such, the SST represents a more exaggerated orthostatic challenge. As the HUT and SST show poor statistical agreement, they should not be used as interchangeable orthostatic challenges.

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RELATIONSHIP BETWEEN MEMBRANE POTENTIAL AND CALCIUM ENTRY IN MYOGENIC CONTRACTION OF SKELETAL MUSCLE ARTERIOLES

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The myogenic response, characterised by the ability of the arteriole to constrict following increased intraluminal pressure or dilate in response to a reduction in perfusion pressure, has been appreciated since early this century. This response is critically important for the development of resting vascular tone, upon which other control mechanisms exert vasodilator and vasoconstrictor influences. The signalling mechanisms fundamental to myogenic contraction will determine which control pathway is of importance in the control of arteriole tone *in vivo*. Clearly, it is pivotal to understand the mechanisms underpinning myogenic tone in order to understand the influences of other control mechanisms. *In vitro* experiments on blood vessels have shown that a change in vascular tone is invariably associated with changes in vascular membrane potential, which can be used to dissect out the components contributing to tone. It has been known for some time that pressure induced myogenic tone is associated with membrane depolarization and that Ca^{2+} entry through voltage operated L-type calcium channels is essential to the development of myogenic tone. Research in this area has proved to be technically challenging, but recently we have clarified some aspects of the relationship between membrane potential and diameter changes in pressurised arterioles.

Cremaster muscles were removed from Sprague-Dawley rats anaesthetized with sodium thiopental (100 mg/kg b.w., i.p.) which were then killed. The muscles were placed in a cooled dissection chamber and a segment of the first order arteriole (passive diameter approximately 160 μm) microdissected from surrounding tissues. Isolated arteriole segments were cannulated on glass micropipettes, pressurised under zero flow and mechanical responses examined using video microscopy. Under these conditions arterioles typically exhibit spontaneous myogenic tone. Membrane potential was recorded using standard electrophysiological techniques. For measurements of smooth muscle Ca^{2+}, vessels were loaded with the Ca^{2+}-sensitive dye fura 2-AM (1 μM) and fluorescence recorded using a photometer based system coupled to the microscope.

Simultaneous recording of vessel diameter with membrane potential revealed that changes in diameter were associated with changes in membrane potential. Increase in intraluminal pressures from 10 to 120 mmHg were associated with membrane depolarization from 58.5 ± 1.6 to 34.3 ± 1.3 mV (n = 8). At selected pressures, blocking the L-type calcium channels with nifedipine (100 nM -10 μM) resulted in loss of myogenic tone and subsequently the arterioles behaved passively. Nifedipine treatment similarly inhibited pressure induced changes in intracellular Ca^{2+}. In contrast, the membrane potential (MP) changes associated with changes in intraluminal pressure were not significantly altered in the presence of nifedipine (see Table).

Pressure (mm Hg)	Control Diameter (μm)	Diameter (μm) in Nifedipine	Control MP (mV)	MP (mV) in Nifedipine
10	74.6 ± 7.5 (9)	88.7 ± 7.2 (11)	57.9 ± 1.5 (9)	56.7 ± 1.7 (11)
70	74.4 ± 5.0 (15)	133.7 ± 9.9 (13)	38.3 ± 1.0 (15)	39.6 ± 1.3 (13)
120	78.6 ± 11.5 (5)	155.6 ± 17.5 (8)	35.3 ± 1.5 (5)	34.3 ± 0.7 (8)

Further evidence for a temporal relationship between membrane potential and the mechanical response was obtained from continuous records in vessels exhibiting spontaneous vasomotion. The results are consistent with the idea that pressure/tension induced changes in membrane potential precedes Ca^{2+} entry via L-type channels.

THE ROLE OF CANNABINOID RECEPTORS AND INTRACELLULAR GAP JUNCTIONS ON EDHF MEDIATED RELAXATIONS IN RAT ISOLATED RENAL ARTERY

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Endothelium derived hyperpolarizing factor (EDHF) is an important mediator of nitric oxide (NO)/ prostacyclin (PGI_2) independent vasodilatation in various vascular beds (Feletou & Vanhoutte, 1988). To date, the chemical identity of EDHF and its mechanism of action has not been fully elucidated, but it may differ between vascular regions and species. Studies suggest that intra-epithelial cell gap junctions and cannabinoid receptors may be involved in EDHF-mediated responses (Triggle *et al.*, 1999).

In this study, we examined the effects of the cannabinoid receptor antagonist SR141716A and the specific gap junction inhibitor GAP 27 peptide on EDHF-mediated responses induced by the muscarinic agonist carbachol in the rat renal artery. Sprague Dawley rats of either sex were killed by asphyxiation using CO_2 followed by decapitation. Segments of the isolated renal artery were set up in a myograph as previously described (Jiang *et al.*, 1998). The preparations were treated with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) and the cyclooxygenase inhibitor indomethacin (10 μM) to eliminate contributions by NO and PGI_2, respectively.

Carbachol induced relaxations were significantly reduced by the large conductance Ca^{2+}-activated K^+ channel inhibitor charybdotoxin (200 nM) and Na^+/K^+/ATPase inhibitor ouabain (30 μM), which is consistent with previous observations on the activity of EDHF in this tissue (Jiang *et al.*, 1998). The cannabinoid receptor antagonist SR141716A (30 μM) and the gap junction inhibitor GAP 27 peptide (300 μM) significantly reduced relaxations to carbachol but not the NO donor sodium nitroprusside (SNP). However, the combination of SR141716A and GAP 27 peptide did not produce a further inhibition of carbachol relaxations to that of either drug alone.

These findings indicate the possible involvement of cannabinoid receptors and gap junctions in EDHF-mediated vasodilatation induced by carbachol in the rat renal artery.

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TYROSINE PHOSPHORYLATION OF MITOGEN ACTIVATED PROTEIN KINASE FOLLOWING ACUTE INTRA-LUMINAL PRESSURE INCREASE OR EPIDERMAL GROWTH FACTOR TREATMENT OF ARTERIOLES

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Skeletal muscle arterioles develop spontaneous tone at steady intra-luminal pressures and demonstrate active myogenic vasoconstriction when exposed to acute increases in intra-luminal pressure. Knowledge of the signalling pathways underlying myogenic tone and vasoconstriction is incomplete, however evidence indicates that the basic mechanism involves Ca^{2+}- entry through voltage gated channels and Ca^{2+}-mediated myosin light chain phosphorylation (Zou *et al.*, 1995). Previous studies have shown that tyrosine phosphorylation of MAP kinase is involved in smooth muscle contraction following agonist stimulation (Adam *et al.*, 1995) and our work showed that a MEK inhibitor, PD98059, reduced tone in isolated, pressurised arterioles (Spurrell *et al.*, 1999). The present studies therefore attempted to specifically demonstrate the tyrosine phosphorylation of p44/42 MAPKs in pressurised rat cremaster arterioles using western blotting and enhanced chemiluminescence (ECL) detection.

Arterioles (active diam: 60-100 μm) were dissected from cremaster muscle of anaesthetised (sodium thiopentone, 100 mg/kg i.p.) Sprague Dawley rats, which were then killed. The vessels were cannulated on micro-pipettes, mounted in a superfusion chamber (vol: 5 mL), positioned on the stage of an inverted microscope and superfused with Krebs bicarbonate buffer (PBS) at 4 mL/min at 34±0.5°C. To enable study of myogenic responses in the absence of shear stress, arteriolar segments were pressurised without intraluminal flow. After being tested for pressure leaks, the passive maximum diameter was determined by exposure to 10 μM acetylcholine at 70 mmHg and this diameter was used as the reference for each experiment. Following agonist responsiveness (phenylephrine 1 μM), and demonstration of the myogenic responses, intra-luminal pressure was reduced to 30 mmHg for 30 min prior to each treatment. Vessels were then subjected to an acute increase in intra-luminal pressure to 100 mmHg for 15 min or treated with epidermal growth factor (EGF: 50 ng/mL) for 10 min.

Vessels, superfused with Ca^{2+} (2.5 mM) containing PBS, demonstrated significant (P<0.05) myogenic vasoconstriction in response to an intraluminal pressure step increase (diam.- 30 mmHg: 69.70 ± 12.76%, 100 mmHg: 39.97 ± 5.29%; n=4). Vessels exposed to Ca^{2+} free PBS and subjected to the pressure step protocol acted passively (diam.-30 mmHg: 78.59±1.25%, 100 mmHg: 112.28±1.23%; n=4). Vessels treated with EGF did not cause significant vasoconstriction, however, they did demonstrate significantly (P<0.05) higher levels of phosphorylated MAPKs, p44 and p42, with respect to vessels subjected to the intra-luminal pressure step protocol (p44-EGF: 1133±178 pixels; n=4, 30 mmHg: 182±43 pixels; n=4, 100 mmHg: 153±63 pixels; n=3). Vessels exposed to Ca^{2+} free PBS and subjected to the pressure step protocols did not demonstrate increased phosphorylated MAPK (30 mmHg: 56±8 pixels; n=2, 100 mmHg: 167±125 pixels; n=2).

EGF stimulated an increase in tyrosine phosphorylation of both p44 and p42 MAPK in cannulated cremaster arterioles. In contrast, increased intra-luminal pressure did not stimulate tyrosine phosphorylation of either p44 or p42 MAPK under the present experimental conditions. As the MEK inhibitor, PD98059, reduced arteriolar tone in earlier studies (Spurrell *et al.*, 1999), other experimental parameters, including different time points, higher pressures, or maintaining increased wall tension, may be required to fully explore the mechanisms underlying arteriolar myogenic responses. Alternatively, the dilation occurring in response to PD98059 may be a result of unrelated mechanisms.

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DEVELOPMENT OF A MOUSE ISOLATED ARTERIOLE PREPARATION FOR STUDY OF THE MICROCIRCULATION

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Studies of the signaling mechanisms underlying the vasomotor functions of the microcirculation are restricted by the amount of tissue available and the need to commonly rely on small molecular weight pharmacological inhibitors that often lack specificity. The increased availability of genetic models, including knock-outs and transgenic animals, provides a new approach to studying the effects of signaling pathways and pathological states on elements of the microcirculation. As these models are typically produced in mice this provides technical difficulties in isolating and cannulating arterioles due to the size of the animal and the vessels available for study. The aim of the present study was, therefore, to develop methods for isolating and cannulating mouse cremaster muscle arterioles for in vitro studies of microcirculatory function. This vascular bed was chosen to complement the in vivo preparation which can be studied by video microscopy in the anaesthetised animal.

Cremaster muscles were removed from anaesthetized (sodium thiopentone 100 mg/kg) BALB/c mice (16-18 g), which were then killed. The muscles were placed in a cooled (4^{o}C) buffer-filled chamber for microdissection of arterioles. Arterioles were microdissected as used in previous studies on rat (Meininger *et al.*, 1991; Hill *et al.*, 2000). Arterioles were then cannulated on micropipettes (tip diameter approximately 40 μm), pressurized to 60 mmHg, superfused (4 ml/min) with a modified Krebs-HEPES solution (pH 7.4, temp. 34^{o}C) and studied by video microscopy (×20 objective, NA 0.75). All preparations were studied in the absence of intraluminal flow to isolate myogenic responses from shear-dependent effects. Myogenic responsiveness was examined by measuring vessel diameter under steady state conditions; intraluminal pressure was randomly set over the range 20-100 mmHg. Additional studies were conducted to examine the temporal relationships between vessel diameter and an acute step change in intraluminal pressure (30-100 mmHg). Vasomotor responsiveness was also examined following application of exogenous adenosine (0.3-1000 μM), acetylcholine (100-0.1 nM) and phenylephrine (0.03-10 μM). Passive arteriole diameter and distensibility characteristics were measured after superfusion with buffer containing 0 mM Ca^{2+} and 2 mM EGTA.

The first branching order arterioles developed spontaneous myogenic tone under pressurized conditions. At 60 mmHg (approximate in vivo pressure) arterioles had an active diameter of 33±3.1 μm (range 25-40 μm; n=5) compared to a passive diameter (obtained under conditions of 0 mM extracellular Ca^{2+}) of 84±3.7 μm. Over the pressure range studied active arteriolar diameter was significantly less than that under passive conditions demonstrating steady-state myogenic reactivity. In response to an acute pressure step (30-100 mmHg) arterioles initially exhibited a passive distension which was followed by vasoconstriction over an approximate 2 minute period. Arterioles showed concentration-dependent dilator responses to acetylcholine (EC_{50} 12.8±0.28 nM) and adenosine (EC_{50} 15.5±1.3 μM) and vasoconstriction to the α-adrenoceptor agonist phenylephrine (EC_{50} 0.769±0.21 μM).

The data indicate that viable arterioles from the mouse cremaster muscle can be isolated and cannulated for studies of microvascular function. As with their rat counterpart they exhibit spontaneous myogenic tone, agonist reactivity and responsiveness to both endothelium–dependent and independent dilators. The availability of the mouse isolated arteriole preparation, in combination with genetic manipulations, has the potential to expand our understanding of microcirculatory control mechanisms at the molecular level.

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a-ADRENOCEPTOR SIGNALLING INVOLVES CYCLOOXYGENASE-1 IN OLD BUT NOT YOUNG RAT AORTA

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α_1-Adrenoceptor stimulation can cause activation of phospholipase A_2 and the release of arachidonic acid (see Zhong & Minneman, 1999) in addition to the classical phospholipase C signalling system. In terms of vascular smooth muscle contraction, the phospholipase C signalling pathway is thought to be more important with only a limited number of studies implicating arachidonic acid and the subsequent metabolites of cyclooxygenase (thromboxane, prostaglandins) as mediators of the contraction (see Connoly *et al.*, 1998). We have shown that the cyclooxygenase-2 inhibitor NS-398 (Futaki *et al.*, 1994) reduced α_1-adrenoceptor mediated constriction in aorta from 54 but not 8 week old rats, presumably through preventing the formation of vasoconstrictor prostaglandins. Furthermore, Western blot analysis showed that there was increased cyclooxygenase-2 protein expressed in aorta from 54 week rats compared to 8 week rats (van der Zypp *et al.*, 2000).

In the present study we examined the effect of the cyclooxygenase-1 inhibitor piroxicam (Laneuville *et al.*, 1994) on α_1-adrenoceptor mediated constriction and also cyclooxygenase-1 protein expression in aorta from 8 and 54 week old rats. Male Sprague-Dawley rats 8 and 54 weeks old were anaesthetised with sodium methohexitone (50 mg/kg i.p) and were then killed by decapitation. The aortae were removed and were mounted as ring segments in organ baths for the study of phenylephrine mediated constriction. In aortae from 8 and 54 week old rats the α_1-adrenoceptor agonist phenylephrine (10^{-9} – 10^{-5}M) produced a concentration dependent constriction. The phenylephrine constriction of endothelium-intact aorta was not significantly different between the two groups with -log EC_{50} values of 6.87 ± 0.18 and 6.69 ± 0.29 and maximum constrictions of 1.60 ± 0.23g and 1.96 ± 0.30g in 8 and 54 week aortas respectively. However the cyclooxygenase-1 inhibitor piroxicam (100 μM) inhibited the phenylephrine constriction in 54 week aortas by 35% but had no effect in 8 week aortas. These data show marked changes in α_1-adrenoceptor signal transduction in the two age groups which may be explained by either increased arachidonic acid release in the older rats or increased cyclooxygenase enzyme activity leading to a greater production of prostaglandins and thromboxanes. This latter suggestion is a possibility as Western blot analysis of cyclooxygenase protein using a rabbit polyclonal antibody and chemiluminescent detection showed cyclooxygenase-1 expression is greater in 54 week aortas compared to 8 week aorta. This adds to our previous findings that cyclooxygenase 2 expression was also increased in the aortas from the older rats.

These data suggest that α_1-adrenoceptor signalling changes in older blood vessels and that this may be due to altered expression of both cyclooxygenase isoforms.

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ORIGINS OF DELAYED ONSET MUSCLE SORENESS

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Eccentric exercise, where the contracting muscles are forcibly lengthened, as occurs in many sports including downhill walking and skiing, is followed by delayed onset muscle soreness (DOMS). These experiments explore the origin of DOMS which is characterized by the absence of pain unless the muscle is stretched, palpated or contracted.

It is known that small myelinated Group III (or Aδ) and unmyelinated Group IV (or C) fibres mediate the pain in skeletal muscle. It is believed that the local release of chemical substances, as a result of the inflammatory process triggered by muscle damage from eccentric exercise, sensitises polymodal nociceptors. The hypothesis is that sensitisation is sufficient to make normally non-painful stimuli such as stretch, contraction or palpation able to generate pain. However, most sensitised nociceptors develop background activity and therefore generate pain continuously (Mense, 1993). That is not the case with DOMS.

To establish whether sensitization of nociceptors takes place during DOMS, mild muscle pain was induced by chemical means, and pain ratings were recorded from human subjects before and after a period of eccentric exercise. Sterile hypertonic (5%) saline (0.2 ml) was injected into the triceps surae muscle. The saline was injected into each leg at comparable sites before the exercise, and after one leg had undergone eccentric exercise. In each leg physiological (0.9%) saline was also injected to act as a control for any mechanical effect from the fluid injection. The eccentric exercise required subjects to walk backwards downhill, on a moving treadmill inclined at ~15°. Subjects walked for 1 hour with a stepping rate of about 30-35 steps/minute. Subjects were asked to use a toe-to-heel action when stepping back to ensure that the triceps surae of the experimental leg was actively contracting while being stretched. The other leg was brought down flat on the treadmill belt, so that its triceps was not stretched or contracted and so it could be used as a control. Forty-eight hours after the exercise the muscle was mapped for sore areas using a compression gauge. It showed that sensitive areas were localised, randomly distributed and not restricted to the muscle-tendon junction.

Soreness evoked by hypertonic saline injected into sore areas of the muscle was less in muscles of subjects experiencing DOMS. Our observation is therefore not consistent with the nociceptor sensitization hypothesis, according to which it might have been expected that the response to the saline stimulus would be greater, not less.

In a second experiment, applying controlled compression to a sensitive area of the muscle showed that the perceived pain was significantly heightened when the compression was combined with 20 Hz and 80 Hz vibration. In an unexercised muscle, vibration had the opposite effect, it reduced any pain. As it is known that nociceptors are typically not responsive to vibration, while large afferents are sensitive to both high and low frequency vibration, this result suggests large afferent involvement. In a third experiment, pain thresholds were measured before, during and after a pressure block of the sciatic nerve. The block affected only the large diameter nerve fibres, as evidenced by disappearance of the H-reflex. Tenderness thresholds rose significantly during the block. Integrity of conduction in small fibres was shown by the absence of any increase in latency for painful heat stimulation. Nor was cold sensation lost in any subject.

It is concluded that large diameter muscle afferents, most likely from muscle spindles, contribute to the sensation of pain following eccentric exercise. While the experiments suggested the involvement of non-nociceptive mechanoreceptors in the generation of DOMS, a contribution from nociceptors has not been excluded.

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INTERACTION OF DIET AND EXERCISE ON SKELETAL MUSCLE ADAPTATIONS AND EXERCISE PERFORMANCE IN RATS

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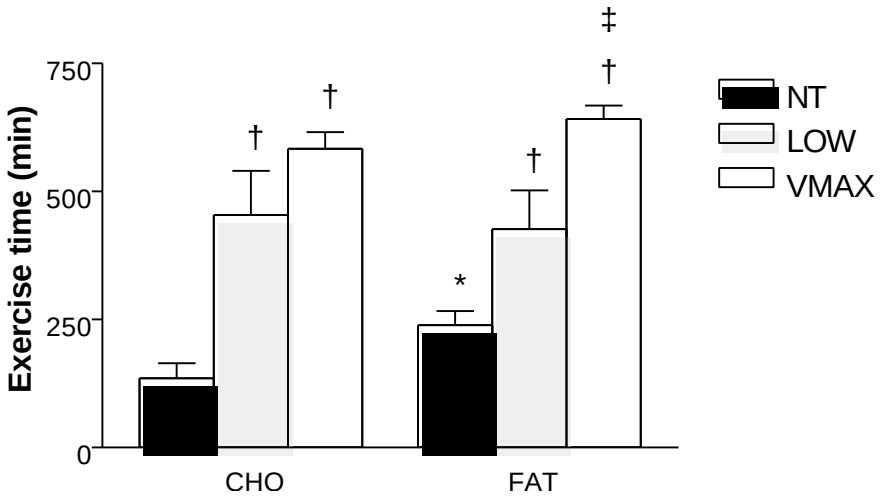
We determined the interaction of different exercise (low- and high-intensity) and diet (high-fat and high-carbohydrate) regimens on selected skeletal muscle and liver adaptations and their metabolic consequences for endurance performance in rats. We hypothesised that a high-fat diet in combination with low-intensity training would evoke the greatest metabolic adaptations for fat metabolism in skeletal muscle and subsequently improve endurance running capacity to a greater extent than when either low or high-intensity training was undertaken on a high-carbohydrate diet.

Sixty-four female Sprague-Dawley rats undertook a baseline treadmill run to exhaustion at 16 m min^{-1} (RUN1) and were then divided into one of two dietary conditions: high-carbohydrate (CHO) or high-fat (FAT). Each dietary group was then divided into one of 3 subgroups: sedentary control that performed no training (NT); low-intensity running (8 m min^{-1}; LOW) and maximal voluntary running speed without electrical stimulation (28 m min^{-1}; VMAX). Training volume was the same for LOW and VMAX (1,000 m $session^{-1}$) and animals ran 4 d wk^{-1} for 6 wk. In order to assess the interaction of the higher intensity training with diet, a second endurance test (RUN2) was undertaken after 6 wk at either 16 m min^{-1} or 28 m min^{-1}. At the completion of the training programme, all animals were anesthetised by intraperitoneal injection of pentobarbital sodium (60 mg kg^{-1} BM). Once the anesthesia took effect, hindlimb muscles from the right leg were exposed and the soleus, the red vastus lateralis and the white vastus lateralis were dissected out, rapidly frozen and stored in liquid nitrogen. The abdomen was then opened and a portion of liver was excised and frozen. All animals were killed by heart removal.



NT ran 77% longer at 16 m min^{-1} after FAT than CHO (239 ±28 vs. 135 ±30 min, $P<0.05$). There were no differences for LOW when rats ran at 16 m min^{-1} (454 ±86 vs. 427 ±75 min for CHO and FAT), but VMAX rats fed fat ran longer than CHO when tested at 28 m min^{-1} (100 ±28 vs. 58 ±11 min, $P<0.05$). There were significant main effects for both diet and training on liver glycogen concentration ($P<0.01$) and main effects of diet ($P<0.01$) on citrate synthase and *b*-hydroxy-acyl-CoA dehydrogenase activity in selected skeletal muscles.

In conclusion, significant metabolic adaptations in skeletal muscle and liver occurred in response to both diet and exercise. However, training intensity rather than volume exerted a stronger influence on subsequent endurance running capacity.

DIFFERENTIAL EFFECTS OF EXERCISE ON THE GENE EXPRESSION OF INSULIN SIGNALLING INTERMEDIATES IN HUMAN SKELETAL MUSCLE

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In recent years, it has become apparent that transcriptional regulation of gene expression is an integral component of skeletal muscle adaptation to exercise. Although the mechanisms by which insulin signalling is enhanced following exercise remain unclear, the gene expression of key proteins involved in the insulin-signalling pathway could be mediated by endurance training and may account for some of the improvements in insulin signalling following chronic exercise. In the present study, we hypothesised that a mechanism contributing to the enhancement of insulin action following endurance training in human subjects is upregulated gene expression of key members of the insulin-signalling cascade.

Seven untrained subjects (4 female and 3 male) completed nine days of cycling at 63 ± 2 % VO_2 peak for 60 min/day. Muscle samples from vastus lateralis were obtained by needle biopsy prior to, immediately after and 3 hours after the exercise bouts (on days 1 and 9). The gene expression of IR, IRS-1, IRS-2 and the p85α subunit PI 3-kinase were determined in skeletal muscle using Taqman PCR analysis. The gene expression of IRS-2 and PI 3-kinase were significantly higher 3 hours after a single exercise bout, although short-term training ameliorated this effect. Gene expression of IR and IRS-1 were not significantly altered at any time point. These results suggest that exercise may have a transitory impact on the expression of IRS-2 and PI 3-kinase; however, the predominant actions of exercise on insulin sensitivity appear not to reside in the transcriptional activation of the genes encoding major insulin-signalling proteins.

THE EFFECT OF EXERCISE TRAINING ON LIPID OXIDATIVE GENE EXPRESSION IN HUMAN SKELETAL MUSCLE

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Endurance training results in an increased capacity to utilize lipids as an energy source. One important adaptation to exercise training is the increased expression of many genes coding for oxidative and structural proteins, yet the mechanisms underlying these adaptations are not known. Increased fatty acid levels following exercise may activate signaling pathways targeted to genes encoding lipid oxidative enzymes, possibly through the activation of the peroxisome proliferator activated receptors (PPARs) family of transcription factors. To examine the impact of moderate intensity exercise training on the expression of genes that code for key lipid-oxidative enzymes, seven healthy, untrained adults (3 male and 4 female; 28.9 ± 8.2 years, VO_2 peak = 37.1 ± 7.1 ml kg^{-1} min^{-1}, mean ± SD) underwent a 9 day cycle ergometer training program of 60 min cycling per day at 63 ± 2% VO_2 peak. On days 1 and 9 of the program, muscle samples from vastus lateralis were obtained by needle biospy before, immediately after, and again 3 hours after the exercise bout. Following 9 days of training total lipid oxidation was significantly increased (19.2 ± 2.8 vs 24.9 ± 1.9 g/60min, mean ± SE, $P<0.01$). Gene expression of fatty acid translocase (FAT/CD36), carnitine palmitoyltransferase 1 (CPT1), PPARα and PPARγ were analyzed using Taqman PCR analysis. The expression of all genes was unaltered immediately and 3 hours following a single exercise bout. Following 9 days of exercise training, FAT/CD36 and CPT I mRNA levels were increased before, immediately after and 3 hours post-exercise bout, on average by 38.2% and 59.6%, respectively ($P<0.05$). Following exercise training, PPARα gene expression was unaltered, while PPARγ mRNA levels were reduced before, immediately after and 3 hours post exercise on average by 34.5% ($P<0.05$). The results of this study demonstrate that in response to exercise training, skeletal muscle adapts to increased fat oxidation by selectively upregulating the gene expression of certain key lipid metabolism proteins. However, the changes in the transcription of these lipid oxidative genes are not accompanied by increased expression of the PPAR transcription factors which have been reported to regulate these genes.

ACTIVATION OF THE GLYCINE RECEPTOR IS ACCOMPANIED BY A STRUCTURAL REARRANGEMENT OF THE M2-M3 LOOP

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The glycine receptor chloride channel (GlyR) mediates inhibitory neurotransmission in the spinal cord and brainstem. Mutations in the extracellular M2-M3 loop of this receptor have previously been shown to uncouple the ligand-binding process from the channel activation gate (Lynch *et al.*, 1997), indicating that this domain comprises an important structural component of the channel activation mechanism. However, it has not yet been shown that this domain moves during channel activation. In the present study, the substituted cysteine accessibility method (Karlin & Akabas, 1998) was used to investigate whether a structural rearrangement of the M2-M3 loop accompanies channel activation. In this technique, native or engineered cysteine residues are probed with a water-soluble, cysteine-specific methanethiosulfonate (MTS) derivative. If a functional property of the channel is irreversibly modified upon exposure such a reagent, the cysteine is assumed to be exposed at the water-accessible surface of the protein. If the rate of reaction of modification varies in a similar manner between the closed and open states for both negatively- and positively-charged MTS agents, the surface orientation of the cysteine residue is assumed to change between closed and open states.

The 11 residues comprising the M2-M3 loop of the human GlyR α1 subunit (R271 to K281) were individually mutated to cysteine. Mutations were constructed using a PCR-based technique and plasmid DNA encoding both wildtype (WT) and mutated subunits were transiently transfected into HEK293 cells. Transfected cells were perfused with a Tyrode's solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 with NaOH. Glycine-gated currents were measured using whole-cell recording from cells voltage-clamped at -40 mV. Patch pipettes contained (in mM): 145 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 EGTA, pH 7.2 with CsOH.

The thiol-modifying agent, MTS-ethyltrimethylammonium ($MTSET^+$), irreversibly modified 5 of the 6 cysteine-substituted residues closest to the M2 end of the loop. Covalent modification of these cysteines resulted in either an increase (R271C, A272C, S273C, K276C) or a decrease (P275C) the apparent glycine affinity, without any effect on the saturating current amplitude. Each of these substituted cysteines reacted more rapidly with $MTSET^+$ in the open state than in the closed state. Two of these residues (R271C and A272C) also reacted more rapidly with the negatively-charged derivative, MTS-ethylmethanesulfonate ($MTSES^-$), in the open state than in the closed state. $MTSES^-$ did not induce any functional change in the S273C or the P275C mutant receptors, and its rate of reaction with K276C was immeasurably fast.

The modification rates of R271C and A272C were slower in the channel closed state than in the channel open state for both a negatively- and a positively-charged MTS derivative. The most likely interpretation of this result is that the respective residues become more exposed to the protein surface as the channel transitions from the closed to the open state. This provides strong evidence for a structural role for this domain in the channel gating process.

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IMPACT OF EXERCISE ON SKELETAL MUSCLE ACUTE GENE EXPRESSION; USE OF GENE-ARRAY TECHNOLOGY

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Skeletal muscle responses to exercise include myofibre hypertrophy, mitochondrial proliferation and numerous changes in transport pathways and cellular metabolism. These changes are the result of complex alterations in the transcription and subsequent translation of many thousands of genes. The investigation of genetic adaptations in skeletal muscle in response to exercise training has so far been limited to individual genes. Such data do not give a true indication of the complex coordinated adaptive transcriptional response following exercise training. Gene array is a relatively low-cost technology that allows for several thousand genes to be analyzed simultaneously. The GF211 filter (Release 1, Research Genetics) contains 8 grids, each with 0.5 ng insert DNA immobilized on a nitrocellulose membrane to which radioactively labeled cDNA from the tissue of interest is directly hybridized. The aim of this study was to analyze the alterations in gene expression following a single exercise bout of 40 minutes in untrained individuals. Five healthy untrained males were subjected to a single sub-maximal exercise trail (72 ± 2% VO_2 peak) for 40 minutes. Muscle samples from vastus lateralis were obtained by needle biopsy immediately before and after exercise. RNA was extracted and a total of 2 μg pooled RNA (0.4 μg/subject) were transformed to ^{33}P-labelled cDNA, heat denatured and hybridized to the membrane. In order to identify those genes whose expression levels changed in response to exercise, a cut-off value was established at the point of visual verification (hybridization intensity ≥ 2000) using Pathways 2.01 software. Of the genes with intensities >2000, over 70% exhibited a decrease in expression following exercise. The data were then further refined by exclusion of genes whose level of expression was altered by less than a 1.5 fold change. Using these criteria, we identified 97 genes whose expression level was altered with exercise. Of these, 13 genes were upregulated while 84 genes were downregulated following an acute exercise bout, with each point visually verified. In this study, individual gene analysis represented an important analysis step, as many values were clearly the result of contamination and hybridization overlap, resulting in false data points. In order to further validate array data, quantitative analysis of selected genes was performed using real time PCR. Primers for TRAMP, TGF-β, RhoE and fibrillarin were designed using PrimerExpress™ and PCR using SYBR Green was performed. TGF-β, RhoE and fibrillarin all displayed decreased expression as indicated by gene array which was confirmed with quantitative PCR, although the absolute fold changes were over-estimated on the gene array. TRAMP appeared to be upregulated on the gene array, however, we were unable to validate this result with PCR. The above results demonstrate an overwhelming genome-wide down-regulation of gene transcription in response to acute exercise. Few genes were selectively upregulated in response to exercise, with further analysis required to confirm the expression of these genes.

GASTRO-INTESTINAL TEMPERATURE AS A MEASURE OF EXERCISING CORE TEMPERATURE

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For research purposes, body core temperature (T_c) is typically measured from a thermistor in the rectum (T_{re}), oesophagus (T_{es}) and/or on or near the tympanum (T_{ty}). These indices can have discordant kinetics and interpretations, although T_{re} and T_{es} are widely used as 'criterion' measures of T_c. Irrespective of validity, both rectal and oesophageal indices suffer problems with subject acceptance and physical restriction of activities. Hence it is appealing to measure T_c from the gastro-intestinal tract (T_{gi}) using ingestible pill telemetry, ie. a pill emitting a temperature-dependent radio signal for telemetric detection. Despite the advantages for using T_{gi}, its validity as a measure of T_c remains equivocal (Kolka *et al.*, 1993; Sparling *et al.*, 1993; O'Brien *et al.*, 1997). The aim of this study was to compare the accuracy of T_{gi} against T_{re} and T_{es}, during upright exercise in differing levels of ambient heat stress.

Twelve physically-active volunteers from the military and civilian sectors of the Australian Defence Organisation participated in this institutionally-approved study (11 males, 1 female; mean ±SD age: 25 ±5 years; height: 173 ±11 cm; weight: 75 ±12 kg). Following two familiarisation trials, subjects exercised in temperate (WARM: 25°C, 60% relative humidity (rh)) and hot (HOT: 35°C, 70% rh) environments, on separate days. These sessions consisted of ~45-min instrumentation and seated rest, 45-min treadmill walking (1.4-1.7 $m \cdot s^{-1}$, 5-10% elevation) to raise T_c to 38.5°C, 15-min load carriage (repeated lift and carry of a 20-kg crate), a second walk of up to 60-min duration (1.4-2.0 $m \cdot s^{-1}$, 5-10% elevation), and 20-min seated recovery. Subjects wore standard Army combat clothing and running shoes, and carried a 20-kg pack during both walks. T_{re} (YSI 401, Yellow Springs Instruments, USA), T_{es} (Edale Instruments, UK) and T_{gi} (CorTemp pill sensor, HTI Inc, FL, USA; BCTM3, PED Inc, USA) were recorded at 1-min intervals throughout sessions. Telemetry pills were ingested with a bolus of water, normally 2-8 hours prior to experimentation.

Mean baseline T_{gi}, T_{es} and T_{re} were 37.3±0.2°C (±SD), 37.0±0.15°C and 37.1±0.20°C, respectively, for both WARM and HOT conditions. T_{gi} was consistently higher (P<0.001) than T_{re} (by 0.20±0.30°C) and T_{es} (by 0.55±0.37°C) throughout the sessions. Linear modelling indicated that T_{gi} was more closely related to T_{re} (n=1914, R^2=0.86, P<0.001) than to T_{es} (n=1586, R^2=0.71, P<0.001), independent of ambient conditions. The mean elevation of T_{gi} above T_{re} remained constant across sessions, ie. they did not uncouple. In contrast, both T_{gi} and T_{re} increased relative to T_{es} across each session (T_{gi}-T_{oes}: 0.2 to 0.9°C; T_{re}-T_{oes}: 0.2 to 0.7°C) as T_c approached the maximum acceptable limit of 39.5°C.

In summary, T_{re} was higher than T_{es} during seated rest, as expected, but T_{gi} was higher than both indices. The small offset between T_{gi} and T_{re} was unaffected by exercising heat stress, whereas both indices were progressively increased relative to T_{es}. Thus, T_{gi} was more indicative of T_{re} than of T_{es} during walking and load carriage.

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IMPORTANCE OF THE CARBOXYL TAIL OF THE ClC-1 CHLORIDE CHANNEL

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The carboxyl tail of the ClC-0 and ClC-1 chloride channels is essential for function. The mutation (R894X) causing myotonia in humans and a mutation (equivalent to A885P in human ClC-1) causing myotonia in the goat both exist in the carboxyl tail (Beck *et al.*, 1996). Co-expression studies using ClC-0 the chloride channel from *Torpedo* electroplax, show that when the transmembrane region of the channel was expressed in *Xenopus* oocytes, no currents were elicited. Currents could be restored by injecting mRNA or protein corresponding to the carboxyl tail (Maduke *et al.*, 1998).

A study, using rat ClC-1 (rClC-1) cDNA expressed in *Sf* insect cells, showed that various truncations of the carboxyl tail either destroyed channel function or the channel functioned normally, depending on the severity of the truncation (Hryciw *et al.*, 1998). The same study showed a region of approximately 18 amino acids to be of functional importance. The truncation at residue L869 (occurring in the hydrophobic domain D13) destroyed function whereas currents were normal when the channel was truncated after residue N895.

Studies were extended to human ClC-1 (hClC-1) and expressed in *Xenopus* oocytes. Equivalent truncations were prepared. Rat and human ClC-1 are 88% homologous, with the greatest variation in the carboxyl tail. Truncations were prepared according to the equivalent amino acid residue, not the equivalent amino acid number. Mutations were incorporated into a hClC-1 cDNA template in the pTLN *Xenopus* expression vector. mRNA was transcribed from linearised cDNA. Oocytes, removed from *Xenopus laevis* anaesthetised using 3 g of 3-aminobenzoic acid ethyl ester in 3 l of water (the *Xenopus* remained anaesthetised during surgery via hypothermia, using ice), were injected with 10-20 ng mRNA. Channel function was analysed using two-electrode voltage clamping 24 h after injection.

The hClC-1 truncation terminating after residue L863 (L869 in rClC-1) elicited no currents as predicted. The hClC-1 truncation terminating at residue N889 also elicited no currents whereas the equivalent truncation after residue N895 in rClC-1 showed normal currents in *Sf* insect cells. The rClC-1 chloride channels (994 residues) is six amino acids longer than hClC-1 (988 residues). The extra amino acids occur earlier in the carboxyl tail. The rClC-1 truncation at residue N895 was made using available restriction sites that changed the reading frame after residue R894. This resulted in three alanine residues after residue R894, before the stop codon. This lengthened the construct by three amino acids. Consequently, the length of the rClC-1 truncation after residue N895 is nine amino acids greater than the hClC-1 truncation at residue N889. If hClC-1 is truncated after residue R894 (human mutation), adding 5 amino acid residues when compared to the truncation at residue N889, small currents are apparent (Meyer-Kleine *et al.*, 1995).

It is hypothesised that the length, of the post D13 region, of the ClC-1 carboxyl tail is important rather than the amino acid constitution.

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REGULATION OF SKELETAL MUSCLE CALCIUM RELEASE CHANNELS BY INORGANIC PHOSPHATE AND CALSEQUESTRIN

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In muscle, intracellular [Ca^{2+}], and hence muscle force is regulated by uptake and release of calcium from the sarcoplasmic reticulum (SR). Inorganic phosphate (P_i) has a profound effect on SR Ca^{2+} handling since it is able precipitate Ca^{2+} within the SR lumen and so keep free [Ca^{2+}] low. P_i accumulates in the myoplasm during exercise and fatigue up to 50 mmol/l concentrations (resting [Pi] 1-5 mmol/l). Calsequestrin (CSQ) is a high capacity intraluminal SR Ca^{2+}-binding protein (~55 kD). It acts primarily as a Ca^{2+} buffer lowering luminal free Ca^{2+}, localising Ca^{2+} for Ca^{2+} release and aiding Ca^{2+} re-uptake. Recent evidence suggests that P_i and CSQ have direct effects on the ryanodine receptor (RyR) in the SR membrane. P_i has been reported to activate RyRs (Balog *et al.*, 2000). CSQ can be dissociated from the SR by increasing the ionic strength in the SR lumen (Ikemoto *et al.*, 1989). CSQ dissociation has been used to show that CSQ can either stimulate (Kawasaki & Kasai, 1994) or inhibit (Beard *et al.*, 1999) RyRs. We have investigated the effects of luminal and cytoplasmic P_i on single RyR channels and how these effects are modulated by CSQ.

SR vesicles were prepared from the back and leg muscles of New Zealand rabbits killed by captive bolt prior to muscle removal. Vesicles were incorporated into an artificial planar lipid membrane separating two solutions: *(cytoplasmic)* 100 nmol/l $Ca^{2+}_{(free)}$ (1 mmol/l $CaCl_2$ plus 4.5 mmol/l BAPTA), 2 mmol/l ATP, 10 mmol/l TES (pH7.4), 230 mmol/l Cs methanesulfonate (CsMS), 20 mmol/l CsCl, 300 mmol/l manitol; *(luminal)* 1 mmol/l $CaCl_2$, 10 mmol/l TES (pH7.4), 30 mmol/l CsMS, 20 mmol/l CsCl.

Increasing luminal ionic strength to 500 mmol/l for ~1 min followed by restoration of control conditions RyRs produced two alternative RyRs responses. Approximately 50% of RyRs (responsive) showed a 6-fold increase in open probability (P_o) that occurred via an increase in channel mean open time (T_o). In the other 50% of channels (non-responsive) no significant change was observed in P_o or T_o. Subsequent addition of CSQ (20 μg/ml) to the luminal bath resulted in a 1.8-fold reduction in P_o of both responsive and non-responsive RyRs. Addition of heat-inactivated CSQ did not produce this inhibitory effect. These results indicate that 1) CSQ could be dissociated from RyRs by increasing the ionic strength on the luminal side of the channel and could be reassociated by addition of purified CSQ to the luminal bath and 2) not all RyRs initially have CSQ bound.

Addition of 10 and 20 mmol/l l luminal P_i had no effect on RyR activity in either the presence of absence of CSQ. Addition of up to 100 mmol/l cytoplasmic P_i produced a slight (20-50%) increase in RyR activity both in the presence of absence of CSQ. The effect of luminal P_i is consistent with previous studies (Balog *et al.*, 2000) but the cytoplasmic P_i effect seen here in the presence of 2 mmol/l ATP was much smaller than that seen previously in the absence of ATP. The possible roles for cytoplasmic P_i in regulating RyRs must be re-evaluated in the light of this new data.

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MHC ISOFORM EXPRESSION AND EC-COUPLING IN MECHANICALLY SKINNED MUSCLE FIBRES OF THE RAT

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The aim of this study was to test the hypothesis that the responsiveness of a mechanically skinned muscle fibre of the rat to T-system depolarization induced activation (an indicator of EC-coupling) is related to its myosin heavy chain (MHC) isoform composition and is independent of the muscle from which the fibre is isolated. This hypothesis was formulated based on a preliminary report by Stephenson *et al.* (1998) that type I fibres from rat soleus (SOL) developed markedly smaller T-system depolarization induced force responses than type II fibres from extensor digitorum longus (EDL).

Adult Long Evans rats (12 – 14 weeks old) were killed by deep halothane inhalation. EDL, SOL and Sternomastoid (SM) muscles were dissected and single fibres were isolated and mechanically skinned under paraffin oil, as previously described (Bortolotto *et al.*, 1999). The fibre segment was mounted on a force measuring system and stretched by 20% of its slack length. After 2 min incubation in a K^+-HDTA (hexamethylenediamine tetraacetate) repriming solution, each fibre preparation was subjected to a series of depolarizations in a Na^+-HDTA solution. Between consecutive depolarizations the fibre was repolarized in the K^+-HDTA solution for 1 min. Under these conditions, the EC-coupling mechanism is impaired and the size of the force responses declines ('fibre run-down') after a number of T-system depolarization induced force responses (Stephenson *et al*, 1999). To ensure that the fibre run-down was not due to a reduction in SR-Ca^{2+} content, each fibre segment was subsequently placed in a solution containing low Mg^{2+} (< 0.03 mM) and caffeine (30 mM) (Fryer & Stephenson, 1996). Finally, the fibre segment was maximally activated in a high Ca^{2+} (pCa < 5.2) solution and then analysed for MHC isoform composition by SDS-PAGE using the Mozdziac *et al.* (1999) protocol with slight modifications.

The maximum depolarization induced force responses (expressed as percentage of the maximum calcium activated force; $\%CaF_{max}$) were significantly (*P<0.05;* ANOVA + Bonferroni's) higher in fibres containing MHC IIb from EDL (61.92 ± 6.25; M ± SEM; *n*=24) and SM (71.99 ± 8.93; *n*=8) than in MHC I SOL fibres (24.98 ± 5.41; *n*=11). By comparison, the number of responses developed by a fibre preparation until the response declined to 25% of its highest level (25% run-down; $R\text{-}D_{25\%}$) was significantly higher in fibres containing MHC IIb from EDL (22.17 ± 2.71; *n*=24) than in fibres containing MHC I from SOL (9.55 ± 0.25; *n*=11) and fibres containing MHC IIb from SM (11.50 ± 1.66; *n*= 8).

Based on these data we conclude that characteristics of EC-coupling in mechanically skinned muscle fibres of the rat are related to both MHC isoform composition and the muscle of origin.

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EFFECTS OF CAFFEINE AND 2-AMINOETHOXY-DIPHENYLBORATE (2-APB) ON THE CONTRACTILE AND ELECTRICAL ACTIVITY RECORDED IN THE PROXIMAL RENAL PELVIS OF THE GUINEA PIG

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It is well established that migrating peristaltic contractions in the mammalian upper urinary tract originate in the proximal regions of the renal pelvis. Circumferentially-cut strips of the renal pelvis display spontaneous contractions which decrease in frequency as strips are taken from more distal regions, the isolated ureter being quiescent in most mammals except man or pig. In this report, we have examined the actions of (i) caffeine, a modulator of Ca^{2+} release channels, (ii) neomycin and 2APB, blockers of the formation and binding of IP_3 to the IP_3 dependent Ca release channel, respectively, and (iii) cyclopiazonic acid (CPA), blocker of the Ca ATPase on the electrical and contractile activity of the guinea-pig proximal renal pelvis.

Strips (2×10 mm^2) of the proximal renal pelvis were excised from guinea pigs humanely killed by stunning and exsanguination. Both the amplitude and frequency of the spontaneous contractions of the strips of proximal renal pelvis were significantly reduced, in a concentration- and time-dependent manner, by 2-APB (60 μM for 60 min, *n*=5) and neomycin (1-4 mM for 60 min, *n*=12). In contrast, caffeine (1 mM for 15-30 min, *n*=6) reduced the amplitude these contractions to 0.28±0.08 (*P*<0.005) of control, without significantly affecting the frequency (0.77±0.17 of control; *P*>0.05). Contraction inhibition was associated with a reduction (50-60%) of the positive iontropic and chronotropic effects of histamine (10 μM for 5 min). Xestospongin C (1 μM for 60 min, *n*=4), another blocker of IP_3 binding, had little effect on the spontaneous contractile activity, even though the responses to histamine were reduced approximately 50%. All contractile activity was abolished in a nominal Ca^{2+} free solution but not significantly affected by BAPTA-AM (50 μM for 30 min, *n*=4), the membrane-permeable chelator of internal free Ca^{2+}. In contrast, CPA (10 μM for >30 min, *n*=15) evoked a rapid increase in contraction amplitude which was associated with a transient increase in contraction frequency (over 20 min), followed by a gradual reduction in contraction amplitude and frequency after 30 min.

Intracellular microelectrode recordings were made in the small portions (2×2 mm^2) of renal pelvis in which the epithelial layer was removed previously by rubbing. Most cells displayed spontaneous nifedipine-sensitive action potentials (Zhang & Lang 1994) (frequency 8.6± 0.97 min^{-1}; *n*=20), consisting of a rapidly-rising initial spike (dV/dt 0.76±0.08 $V.s^{-1}$; *n*=22), followed by a few small oscillations on a long plateau phase (half amplitude duration 2.6±0.5 s; *n*=12) and a 'diastolic' after-hyperpolarization. Some cells also displayed regular depolarizing transients, which failed to reach threshold for action potential discharge. Two microelectrode recordings revealed that these cells could be tentatively designated as 'driving' the cells with more quiescent 'diastolic' membrane potentials. The repetitive spikes on the plateau phase of 'driven' action potentials recorded in larger segments of renal pelvis (Zhang & Lang 1994) or ureter were not present in these smaller, stretched, epithelium-free preparations.

Application of caffeine (0.5 mM, *n*=4), neomycin (1 mM, *n*=6) and 2APB (10-60 μM) produced a time-dependent change in the time course of the spontaneous action potential discharge associated with a truncation of the plateau phase. Caffeine (1 mM, *n*=8) and 2-AP (60 μM, *n*=8) abolished all electrical discharge (within 6 min) in only 40% of preparations. Remaining preparations displayed a resistance to these agents, displaying smaller electrical discharges with truncated plateau phases. CPA (10 μM for 10-20 min, *n*=5) significantly increased the half-amplitude duration 3.63±0.74 fold (*P*<0.01) of all cells in the proximal renal pelvis, without significantly altering the rate of rise of the initial spike or the frequency of discharge (*P*>0.05).

In conclusion, action potential discharge and contraction in the guinea-pig proximal renal pelvis depend on a rise in internal Ca^{2+} arising from both the entry of Ca through L-type Ca^{2+} channels and the release of Ca^{2+} from internal IP_3 dependent stores.

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EFFECT OF TEMPERATURE IN THE UPPER PHYSIOLOGICAL RANGE ON THE CONTRACTILE APPARATUS OF RAT SKELETAL MUSCLE

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It is well known that when isolated intact mammalian muscle preparations are exposed to temperatures higher than 35ºC, force production is markedly depressed and this reduction is in some cases irreversible (Lännergren & Westerblad, 1986). As a result of this, most physiological studies on isolated mammalian muscle preparations are conducted at temperatures that are well below the body core temperature of mammals (37ºC). A study on isolated rat fibres exposed to temperatures ranging from 37ºC-70ºC suggests that isolated muscle cells remain in an apparently relaxed state at temperatures above the normal physiological range when severe disruption of membrane structure is known to occur (Bischof *et al.*, 1995). Since disruption of the sarcolemma would be expected to cause Ca^{2+} entry and contraction, this observation suggests that the contractile apparatus *per se* may not be able to become activated and develop force as the temperature increases above a certain level.

Here we examine the effect of temperature up to 46ºC where muscle necrosis occurs (Seese *et al.*, 1998), on the contractile apparatus of rat skeletal muscle and test the hypothesis that there is a protective mechanism that reversibly reduces the ability of the contractile apparatus to develop force following exposure to high temperature.

Long-Evans Hooded Rats were killed by an overdose of halothane. Extensor digitorum longus (EDL) muscles were dissected and immediately exposed to different temperatures. After exposure, single fibres were mechanically-skinned under paraffin oil, mounted on a force transducer and placed in a relaxing solution. For each individual fibre the maximum Ca^{2+}-activated force and force-pCa curves were obtained using Ca^{2+} buffered solutions (Stephenson & Williams, 1981).

After exposure to 43ºC for 30 minutes and recovery in paraffin oil at 22ºC, maximum force showed initially a marked decrease (90% of initial maximum force) over the first 45 minutes after exposure. Maximum force then recovered to 65% of initial maximum force 100-120 minutes after exposure. Muscles exposed to 43ºC in a physiological solution for 30 minutes showed the same trend as the muscles exposed in paraffin oil. Muscle exposure to 46ºC for 5 minutes in paraffin oil, caused maximum Ca^{2+}-activated force to decrease 80% and this was followed by 80% recovery after 120 minutes. In all experiments the sensitivity to Ca^{2+} expressed as the pCa ($-\log_{10}[Ca^{2+}]$) value corresponding to 50% maximum Ca^{2+}-activated force was not affected by the various temperature treatments but the associated Hill coefficient showed a similar trend as the maximum force responses.

Thus, it appears that force generation to reversibly decrease and force pCa curves become shallower as the temperature is increased in the upper physiological range of temperatures. Such a mechanism is likely to provide protection against the muscle contracting and producing more heat as the temperature approaches the threshold for irreversible damage (muscle necrosis).

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CORRELATION BETWEEN SMOOTH MUSCLE ELECTRICAL ACTIVITY AND SPATIO-TEMPORAL MOTOR EVENTS IN THE GUINEA-PIG SMALL INTESTINE

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Inhibition of prostaglandins synthesis increases spontaneous rhythmic motor activity in the guinea-pig small intestine (Bennett *et al.*, 1976, Maggi *et al.*, 1994). Using a new spatio-temporal method to study intestinal motor events (Hennig *et al.*, 1999), we have shown that cyclooxygenase inhibition, with indomethacin, piroxicam or meclofenamic acid, induces spontaneous contractions (Costa *et al.*, 1999a, b) with a maximal frequency similar to that of slow waves recorded in vivo (Galligan *et al.*, 1985) or in vitro (Smith, 1989). These contractions propagated both orally and aborally over a range of distances. In order to establish whether these contractions are due to action potentials triggered by slow waves, we combined extracellular electrical recording of smooth muscle activity and the spatio-temporal maps video recording technique. Segments of guinea pig ileum, 6-7 cm long, taken from adult guinea-pigs killed humanely by cervical dislocation and bleeding, were incubated in Krebs solution at 37ºC and continuously bubbled with carbogen. The segments were maintained distended to 60% of the threshold for peristalsis. Extracellular recordings were made with floating suction electrodes applied to the serosal surface. Spatio-temporal maps of intestinal diameter changes were constructed from simultaneous video recordings (Hennig *et al.*, 1999).

With combined piroxicam (50 µM) and tetrodotoxin (0.6 µM) slow waves without action potentials were observed with a frequency of 21.5 ± 0.6 cpm (n = 4). The corresponding spatio-temporal maps showed no associated mechanical activity. From 60 to 150 min after the addition of the drugs, slow wave frequency was unaltered, but action potentials occurred irregularly on about 1/3 of slow waves (37 ± 3.3%). Motor activity in spatio-temporal maps occurred in irregular bouts, comprised of narrow rings of segmenting contractions and contractions which propagated orally or aborally. The narrow rings occupied 1.47 ± 0.28 mm of intestine (n = 4), with some being as short as 1 mm. Propagated contractions appeared to be made of sequences of narrow contractions. All contractions were preceded by action potentials occurring 1310 ± 434 (SD) ms before the peak of contraction. The shortest delay between action potentials and the beginning of circular muscle shortening was 569 ± 405 (SD) ms. The duration of contractions (at half peak amplitude) was 9.86 ± 3.24 s for the propagating contractions and 8.41 ± 1.36 s for narrow contractions. After nicardipine (2 µM) action potentials disappeared. The frequency, but not the amplitude, of slow waves decreased from 21.8 ± 0.8 cpm to 18.8 ± 1.3 cpm.

Contractions induced by inhibition of prostanoid synthesis are due to the appearance of slow waves in the circular muscle. These control the timing of action potentials, which occur in discrete, non-propagating narrow rings of circular muscle. Propagating contractions are probably due to sequential activation of such discrete units by the underlying slow waves.

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DIFFERENTIAL MODULATION OF INTRACORTICAL INHIBITION DURING SELECTIVE ACTIVATION OF AN INTRINSIC HAND MUSCLE

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Intracortical (IC) inhibitory circuits in human motor cortex can be studied using transcranial magnetic stimulation (TMS) (Kujirai *et al.*, 1993). A conditioning TMS stimulus that is subthreshold for a motor response activates an IC inhibitory circuit and reduces the size of the muscle evoked potential (MEP) elicited by a suprathreshold test TMS stimulus delivered up to 6 ms later. Inhibitory circuits activated by TMS may be part of a premotor network which selects the appropriate corticospinal neurons for the motor task, and hence play a role in targeting corticospinal outflow to different motoneuron pools for fractionated activation of individual muscles. In the present study we have examined evidence for this proposal as subjects perform selective activation of one of three intrinsic hand muscles.

Seven subjects (4M, 3F) have been tested to date. Surface EMG recordings were obtained from abductor pollicis brevis (APB), first dorsal interosseous (FDI), and abductor digiti minimi (ADM) muscles of the left hand. The hand was secured in a manipulandum and the thumb placed in a ring attached to a load cell which measured thumb abduction force. TMS was applied as subjects performed three tasks with the aid of visual feedback of EMG and force. These were: relaxation of all target muscles (task 1), relaxation of FDI and ADM with isometric thumb abduction of 1N (task 2) or 3N (task 3). Single or paired TMS (Magstim 200, 90 mm circular coil) were delivered randomly for a block of 25 trials ($< 0.2\ s^{-1}$), and MEPs averaged by computer. Conditioning-testing interval was 3 ms. Conditioning TMS intensity was below motor threshold in active APB, and adjusted to produce ~60% suppression of the MEP in APB with all muscles at rest.

Average rectified EMG levels in the pre-stimulus period did not change significantly with task in FDI or ADM (ANOVA, $P > 0.05$), indicating that the subjects were able to keep these muscles inactive while activating APB. Conditioning TMS suppressed the MEP to a similar degree in all muscles at rest. When APB was active during the 1N and 3N contractions, conditioning TMS produced significantly less suppression of the APB MEP compared with the resting state (Tukey's *t*-test; $P < 0.001$) (cf. Ridding *et al.*, 1995). For APB, mean (± SE) size of conditioned MEPs were 35 ± 6% of unconditioned responses at rest, 70 ± 7% with 1N thumb abduction and 76 ± 5% with 3N thumb abduction. In contrast, activation of APB did not influence the effectiveness of conditioning TMS in the relaxed FDI and ADM muscles (ANOVA, $P > 0.05$). For FDI, conditioned MEPs were 32 ± 6% of unconditioned responses at rest, 49 ± 8% with 1N thumb abduction and 44 ± 5% with 3N thumb abduction. Corresponding values for ADM were 37 ± 7%, 38 ± 6% and 48 ± 8%, respectively.

These data support the hypothesis that IC inhibition of corticospinal neurons controlling APB is selectively suppressed when APB is activated in isolation from FDI and ADM. This disinhibition would promote corticospinal activation of APB without overflow of the excitatory command to FDI and ADM. We conclude that differential modulation of IC inhibition contributes to the ability of the corticospinal system to produce fractionated activation of intrinsic hand muscles.

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TIME-COURSE OF EFFECTS OF LIGNOCAINE IN CUTANEOUS TISSUES OF MAN: A LABORATORY FOR FIRST YEAR MEDICAL STUDENTS

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Definitive studies on the time-course of placebo and different dose effects of local anaesthesia for cutaneous tissues in man are not available. The aim of the present studies was to inform first year medical students of the expectations of local anaesthesia for skin suturing procedures in man with particular reference to placebo effects, and to the time-course of effects of different concentrations of lignocaine used clinically. It was postulated that effective local anaesthesia for suturing skin wounds would be present after waiting 10 min following subcutaneous injection of 0.5% (plain) lignocaine and that the effective time for suturing would last a further 15 min. Larger concentrations would be effective at 10 min, and would be effective for suturing for a further 20 min.

The study was approved by the Human Ethics Committee of the University of Newcastle and was carried out as a double-blind trial annually with volunteer students. The students each year were naïve to the processes of the class and by guided discussion designed the appropriate experimental protocol. The class arranged themselves into groups of 4 students, namely a subject, a stimulator, a timer and a recorder. The subject had 4 test sites identified. There were 2 on the flexor surface of each forearm, which were thoroughly cleaned with alcohol. Each site was then injected with one of 4 unknown solutions. These were saline vehicle, 0.5%, 1%, or 2% lignocaine. Each site was then tested with a sharp pin for sensation by comparing the sensation at the injection site with the sensation immediately adjacent but outside the injection site. The responses were recorded as no effect of pinprick (0), modified sensation to pinprick (1), or normal sensation to pinprick. The times tested after injection were at minute intervals for 5 min, and then at 5 min intervals up to 20 min. The data were entered on an Excel spreadsheet and subjected to ANOVA analysis and graphed. The results were brought back to the class in a subsequent class discussion about the mechanisms of local anaesthesia and the interpretation of the data. The present results were obtained from 13 years of experimentation where in each year at least 8 students volunteered.

The data suggest that there is a significant placebo effect at 1 min, which lasts a further 19 min. The degree of sensory modification however, is moderate and would not be suitable for inserting sutures in skin. The data also suggest that 0.5%, 1% and 2% lignocaine causes greater sensory modification than placebo effects to pinprick from 1- 4 min, but would not be suitable for suturing. On the other hand, both 1% and 2% lignocaine strongly modify the sensation to pinprick by the same amount by 4 min after injection (Pdiff 0.5% vs. 1% and 2% < 0.05). However, while 1% and 2% concentration might be suitable for suturing, the effects of both are likely to be available for only a further 10 min. There appears to be no advantage in using 2% rather than 1% concentration.

CHARACTERISATION OF MORPHOLOGICALLY DISTINCT REGIONS IN THE MOUSE CRISTA AMPULLARIS

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As part of ongoing research into the mechanisms underlying the sense of balance, we are characterising the morphological organisation of vestibular sensory neuroepithelium in mice. We are using Scanning Electron Microscopy to quantify hair cell density, together with other characteristics of the *crista ampullaris*.

Mice were painlessly killed with a lethal intra peritoneal Nembutal injection (120 mg/kg) followed by decapitation. Under a stereomicroscope, vestibular organs were dissected free in ice-cold Ringers solution. Before fixation, the enlarged region or ampulla, from each of the six semicircular canals (three per side), were "de-roofed" using micro scissors and forceps. The ampullae, containing the cristae, were then prepared for SEM using the Osmium-Thiocarbohydrazide-Osmium (OTO) method. After dehydration in alcohol, the ampullae were then dried using chloroform vapour as a simple but effective alternative to critical point drying.

Scanning electron micrographs of the ampullary sensory epithelium has revealed two distinct regions: 1) Two tapered hemi-cristae separated by; 2) a narrow raised central zone or *torus*. A single hemi-crista is approximately 110-115 μm in length (longitudinal axis). Transversely, the hemicrista tapers from 90-100μm from its widest to 45-55 μm at the narrowest point. In total the crista possesses in excess of 1200 individual hair cells, each of which has a diameter of approximately 2 to 4 μm. Hair cells are responsible for the conversion of mechanical stimuli to electrical impulses and as such are considered the transduction units of the ampullary crista. The torus, in contrast, is approximately 30-35 μm wide (longitudinal) by 80 μm long (transverse) and appears to be non-sensory since it is devoid of hair cells.

From these preliminary results we see two distinct regions in the mouse crista ampullaris: sensory and non-sensory. The sensory region, consisting of two hemicristae and their associated hair cells and presumably play a role in signal transduction. The second region, the non-sensory torus, lacks hair cells and its function is as yet unknown.

THE IMPACT OF CHANGES IN PROPRIOCEPTIVE INPUT FROM THE NECK ON LOWER LIMB MOTONEURON EXCITABILITY

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Proprioceptive inputs from the neck are known to make important contributions to the complement of reflexes used to maintain postural stability. One of these reflexes is the *tonic neck reflex (TNR).* Together with vestibular reflexes they act to maintain a stable posture by adjusting extensor muscle tone with changes in head and neck posture. The aim of this study was to isolate proprioceptive inputs from the neck (from other sensory systems involved in maintaining postural stability, notably vestibular afferences) and examine whether an alteration in proprioceptive inputs was capable of modulating the amplitude of the Triceps Surae Hoffmann (H) reflex in Humans.

Neck proprioceptive inputs were isolated by stabilising the subject's head and rotating the body around the stabilised head - referred to as longitudinal body rotation against a steady head. The triceps surae H reflex was used as a measure of lower limb motoneuron excitability. Phasic stretch reflexes are prone to spontaneous fluctuations in amplitude. The procedure of triceps surae muscle conditioning was used in order to systematically alter the amplitude of the test triceps surae phasic stretch reflex (Gregory *et al.*, 1987). Muscle conditioning is a procedure which systematically alters the movement and activation history of the muscle. This in turn alters the tonic output from the muscle spindle which in turn alters the amplitude of the reflex response. (Gregory *et al.*, 1990) This test reflex was then used to determine the effect of changing proprioceptive inputs from neck structures on the size of the conditioned triceps surae H reflex.

The current experiments used the following protocol. It was first established that each subject demonstrated a consistent change in H reflex size after conditioning the right triceps surae muscles at a length longer than and shorter than the test length. The right triceps surae muscles were then conditioned at a length shorter than the test length at which the H reflex was elicited. This was deemed to be the control (hold-short) reflex. After a second hold-short muscle conditioning sequence, the seated subject was rotated 60 degrees to the right while their head was maintained in a "straight ahead" position. In this position the H reflex was again elicited.

The results showed that after right longitudinal body rotation with the head maintained in a "straight-ahead" position, the hold-short H reflex was consistently larger than after the control hold-short conditioned H reflex. These findings support the proposal that alterations in proprioceptive inputs from the neck induced by longitudinal body rotation against a head maintained in a "straight-ahead" position are capable of modulating lower limb motoneuron excitability.

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IDENTIFICATION, ORIGIN AND DEVELOPMENT OF THE INTERSTITIAL CELLS OF CAJAL

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The interstitial cells of Cajal (ICC) were first described over 100 years ago. However, for many years their function and embryological origin were unknown, and their identification was equivocal without using electron microscopy. The most significant discovery in elucidating the role of ICC was the finding that ICC express the receptor tyrosine kinase, Kit, and that mice with mutations in the gene encoding Kit have defects in their ICC networks. ICC are found closely associated with the external muscle and nerve fibres in a number of different locations within the gut. Although all ICC appear to express Kit, the different sub-populations differ in their properties and functions. The 2 main populations are those associated with the myenteric plexus (ICC-MP) and those associated with the circular muscle (ICC-CM). Because ICC are closely associated with both nerves and muscle, it was unclear whether they arise from the mesoderm (like muscle) or from the neural crest (like enteric neurons).

To examine this question, pregnant mice were killed by cervical dislocation at a stage at which the embryonic neural crest cells have yet to colonise the gut. The embryos were removed and segments of their gut were then grown in organ culture or under the kidney capsule of adult host mice for 1-2 weeks. When the explants were processed for immunohistochemistry using antisera to Kit to identify ICC, although there were no neural crest-derived cells (neurons or glial cells) in the explants, ICC were present. This indicates that ICC do not arise from the neural crest and, importantly, that ICC do not require the presence of enteric neurons for their survival or differentiation. The ligand for Kit is stem cell factor (SCF). Previous studies have shown that enteric neurons are one of the main sources of SCF, but not all enteric neurons express SCF. We examined which functional classes of ICC express SCF and found that the main source of SCF are nitric oxide synthase (NOS)-immunoreactive neurons, most of which are inhibitory circular muscle motor neurons. However, the vast majority of cholinergic neurons, which are the major excitatory neurons to the circular muscle, did not express SCF.

INITIATION OF SLOW WAVES BY ICC-MY IN THE GUINEA-PIG GASTRIC ANTRUM

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The wall of the gastrointestinal tract generates rhythmical contractions in the absence of external stimulation. Contractions occur at low frequencies, some 2 to 20 beats per minute, depending upon region and species. Each contraction is associated with a long lasting wave of depolarization, termed a slow wave. In the mouse intestine it has been shown that in mutants devoid of myenteric interstitial cells of Cajal, ICC-MY, slow waves are absent (Ward *et al.*, 1994). More recently it has been shown that in mutant mice devoid of inositol 1,4,5 trisphosphate (IP_3) type 1 receptors, slow wave activity is similarly absent (Suzuki *et al.*, 2000). Together these observations suggest that slow wave activity is initiated by ICC-MY and that IP_3 formation is in some way involved.

This presentation will discuss the role of ICC-MY in generating slow waves in the gastric antrum. Using conventional intracellular recording techniques, three distinct types of cell were identified on the basis of their electrophysiological and anatomical properties. Smooth muscle cells lying in the longitudinal layer generated long lasting waves of depolarization with associated bursts of action potentials. ICC-MY generated large amplitude long lasting waves of depolarization, termed driving potentials. Driving potentials consisted of an initial rapid rising phase followed by a more prolonged plateau phase. The plateau phase was triggered after a delay of about one-second, perhaps indicating that its generation involved the formation of a second messenger. Smooth muscle cells lying in the circular layer generated conventional slow waves, these consisted of an initial component and a secondary regenerative component. Paired simultaneous recordings from either ICC-MY and circular smooth muscle cells or ICC-MY and longitudinal muscle cells indicated that the onset of individual driving potentials preceded the depolarizations detected in the adjacent muscle layers.

Together the observations show that slow wave activity in the gastric antrum is initiated by ICC-MY.

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CELLULAR RHYTHMICITY IN ISOLATED SINGLE BUNDLE STRIPS FROM THE CIRCULAR MUSCLE LAYER OF THE GUINEA-PIG GASTRIC PYLORUS

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Electrophysiological investigations of an electrical rhythmicity referred to as slow waves (SWs) in fine single bundle strips from circular smooth muscle layer of the guinea-pig indicate that each SW is composed of a pacemaker and triggered regenerative depolarization. Importantly, both these activities are generated through Ca^{2+} release from inositol 1,4,5 trisphosphate (IP_3) receptor-operated Ca^{2+} stores, which in turn generates inward currents across the plasmalemma. Rhythmicity is likely to occur by a synchronization of local Ca^{2+} release events measured as spontaneous transient depolarizations with pacemaker frequency determined by properties such as the release/refill cycle of Ca^{2+} stores. Sufficient Ca^{2+} release generates CICR, which is regenerative resulting in full SWs.

There have been many reports indicating that gastrointestinal pacemaking is effected by specific cells referred to as Interstitial Cells of Cajal (ICCs). Extramuscular ICCs form plexuses in regions such as the myenteric plexus but intramuscular ICCs are also present within both longitudinal and circular visceral smooth muscle bundles. ICCs form gap junction contacts with other ICCs and with smooth muscle. Evidence that ICCs are likely to act as pacemaker cells underlying SWs is provided by the finding that mutation of the proto-oncogene *c-kit* blocks development of interstitial cells and SWs in murine intestine. There is also evidence that single isolated ICCs exhibit SW-like rhythmicities, though single isolated intestinal smooth muscle cells have also been reported to show such spontaneous rhythmicity, both rhythmicities likely to arise through rhythmical Ca^{2+} release from inositol 1,4,5 trisphosphate (IP_3) receptor-mediated Ca^{2+} stores.

The relative roles of intramuscular ICCs compared to smooth muscle cells as pacemakers in generation of SWs in the single bundle visceral smooth muscle strips is not known. This issue is of importance as pacemaking has generally been attributed to extramuscular networks of ICCs. What is known is that intramuscular ICCs are present within visceral muscle bundles including muscle bundles of the guinea-pig pylorus and are likely to subserve a role in inhibitory neurotransmission. A contraindication to this ICC subclass exhibiting a pacemaker role is that their presence does not universally correlate with SWs as at least one visceral tissue, the gastric fundus, has intramuscular ICCs but does not exhibit SW rhythmicity.

This presentation will review data on SW rhythmicity in single bundle pyloric strips and will discuss a possible role of intramuscular ICCs in this process.

ICC AS TARGETS FOR INNERVATION IN THE GASTROINTESTINAL TRACT

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Over the last century numerous morphological studies have identified a specialized population of cells, known as interstitial cells of Cajal (ICC), within the tunica muscularis of the gastrointestinal tracts from a variety of species (Sanders, 1996). Three functions for ICC in the gastrointestinal tract have been proposed. (i) ICC may act as generators of slow waves. (ii) ICC may facilitate propagation of electrical events along and around the organs of the gastrointestinal tract. (iii) ICC may serve as intermediaries in enteric neurotransmission between nerve varicosities and smooth muscle cells. This talk will review the role of ICC as "targets for neurotransmission within the gastrointestinal tract."

Using immunoelectron microscopy, specialized neuroeffector contacts between enteric varicosities and ICC within the circular and longitudinal muscle layers in the murine gastric fundus and colon and between ICC and nerve terminals within the deep muscular plexus (ICC-DMP) in the guinea pig small intestine have been demonstrated. The implied functional role of ICC in neurotransmission has been supported by studies showing that the loss of muscular ICC (ICC-IM) in the W/Wv mutant stomach results in loss of both cholinergic and nitric oxide-dependent neurotransmission (Burns *et al.*, 1996; Ward *et al.*, 2000). These data suggest that certain populations of ICC express the structural and molecular components that facilitate neurotransmission and that they are heavily innervated by excitatory and inhibitory enteric motor neurons.

A complete understanding of neuroeffector mechanisms in the gastrointestinal tract will require detailed investigations of the structural relationship between ICC and nerves, a molecular analysis of specific receptors and ion channels expressed on ICC and how the activation of receptors and subsequent down stream signaling cascades differ from those found in adjacent smooth muscle cells.

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'CURRENT' ADVANCES IN MECHANICALLY-SKINNED SKELETAL MUSCLE FIBRES

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In skeletal muscle, excitation-contraction (E-C) coupling describes a cascade of cellular events initiated by an action potential (AP) at the surface membrane which ultimately results in muscle contraction. Within a muscle fibre, there are a number of factors that influence and control E-C coupling. Being able to specifically manipulate these factors has proved challenging. One of the simplest methods of gaining access to the intracellular environment of the muscle fibre is to physically remove (mechanically skin) the surface membrane (Natori, 1954). In doing so the myoplasmic environment is opened to external manipulation. Surprisingly, despite the absence of a surface membrane, the ability to activate mechanically-skinned muscle fibres by generating an AP is still possible. Recently, Posterino, Lamb & Stephenson (2000) showed that APs can be triggered in the sealed t-tubules of skinned fibres by electric field stimulation resulting in the generation of twitch and tetanic force. This proved that all the key steps in E-C coupling are retained in this preparation. By using this technique, it is now possible to easily manipulate the myoplasmic environment and observe how altering individual factors affects the normal E-C coupling sequence. The role of important factors such as the redox state of the cell, parvalbumin, the sarcoplasmic reticulum Ca^{2+}-ATPase and free [Mg^{2+}] on twitch and tetanic force can now be specifically addressed independent of other factors.

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Posterino, G.S., Lamb, G.D. & Stephenson, D.G. (2000) Journal of Physiology, 527, 131-137.

THE POWER OF SINGLE CHANNEL RECORDING AND ANALYSIS

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In muscle, intracellular calcium concentration, and hence muscle force and cardiac output, is regulated by uptake and release of calcium from the sarcoplasmic reticulum (SR). These Ca^{2+} fluxes often take place in the presence of a changing cytoplasmic milieu during episodes of metabolic challenge such as seen during hypoxia, ischaemia and fatigue. The ryanodine receptor (RyR) forms the calcium release channel in the SR. In addition, the movement of other ions (eg K^{+} and inorganic phosphate, P_i) across the SR membrane strongly affects the ability of the SR to release calcium.

Calcium release through RyRs is modulated by a wide variety of endogenous molecules including small diffusible ligands such as ATP, Ca^{2+}, Mg^{2+} and protons (pH). Abnormal functioning of RyRs, or their inappropriate regulation in response to altered ligand concentrations during abnormal metabolism, have been shown to produce the changes in contractility that occur in muscle fatigue, ischaemia and myopathies such as malignant hyperthermia (MH) and myoadenlyate deaminase deficiency (MDD). The physiological role for the SR anion channels in muscle is not known nor is it understood why several, differently regulated anion channels are needed in the SR.

The regulation calcium release from the SR is a complex interplay of many regulation mechanisms. In order to tease out and identify the various mechanisms it is necessary to functionally characterise reduced systems (e.g. an isolated RyR or anion channel) and then systematically piece together the rest of the physiological machinery. To this end SR vesicles are incorporated into artificial planar lipid bilayers. Information about the mechanisms underlying regulation of RyRs and anion channels by ATP, Ca^{2+}, Mg^{2+} and pH can then be extracted from single channel recordings using sophisticated signal analysis techniques. These studies have provided new insights into the molecular basis of muscle fatigue, MH and MDD.

Though single channel recording methods (eg. bilayer and patch-clamp) provide a powerful way of studying channel function in exquisite detail, obtaining data can be slow and tedious. This is partly because the membrane systems used to study ion channels are extremely fragile which severely limits the experimental manipulations one can perform. Also, obtaining a specific type of ion channel in a membrane is “hit-and-miss” since there is no way of controlling the number or type of ion channels that either incorporate into a bilayer or that appear in a membrane patch. In recent years new, robust membrane systems have been developed for biosensor applications which may provide much more convenient system in which to study ion channels.

GENE TRANSFER: MANIPULATING AND MONITORING FUNCTION IN CELLS AND TISSUES

Rekha G. Panchal, Angela M. Reilly, David N. Bowser, Jameel Khan, Peter D. Kitchener*, David A. Williams and Steven Petrou, Department of Physiology and *Department of Anatomy and Cell Biology, The University of Melbourne, Melbourne, Vic. 3010.*

The ectopic expression of genes has proved to be an extremely valuable tool for biologists. The most widely used systems involve electrically or chemically mediated transfer of genes to immortalized cell lines. At the other end of the spectrum there are transgenic animal models. As would be expected, there are compromises to be made when employing either of these broad approaches. Immortalised cell lines have limited "physiological relevance" and transgenic approaches are costly and out of the reach of many laboratories. There is also significant time required for the *de novo* generation of a transgenic animal.

An approach in use in our own laboratory offers an additional choice. We have employed two viral transfer systems, Sindbis virus and Adenovirus to transiently express genes and biosensors in whole tissues and freshly isolated cells. Infected cells and tissues appear to retain functional characteristics, and can be used in either short or long-term culture to study cellular mechanisms in a "physiologically relevant" biological background.

To date we have successfully infected cardiac myocytes with recombinant adenovirus encoding GFP, "cameleons" as Ca^{2+} biosensors and the sarcoplasmic/endoplasmic reticulum calcium-ATPase (Serca2a) gene (Reilly *et al.*, 2000). This approach has allowed investigation of the Ca^{2+}-handling deficiencies evident in cardiac hypertrophy and failure, and has also provided a means for further study of the excitation-contraction coupling process of cardiac tissue through specific manipulation of the cardiac sarcoplasmic reticulum. We have also introduced genetically encoded targeted Ca^{2+} sensors into whole hearts and brain slices tio study organelle Ca^{2+} dynamics. To investigate the mechanism of macrophage/microglial migration embryonic phagocytes have been infected with a GFP encoding virus and co-cultured with brain slices to track the movement of labelled cells. We are exploiting the natural tropism of the Sindbis virus to transfer genes of interest to neurones in hippocampal brain slices. Genetically modified Sindbis viruses, created that reduce toxicity in organotypic brain slice culture, are in use to study the Ca^{2+} dynamics of *in situ* single neurones.

A current limitation of viral transfer in brain slices is that most experiments are performed in very young rat pups and slices have been in culture for 14 days or more. Thus, not only is neo-natal brain lacking "adult" neural connectivity, the extended culture results in further dramatic changes in neuronal morphology and networking. For these reasons such organotypic cultures are of limited use. Our long term goal is to achieve viral mediated gene transfer into brain slice cultures of 2 week old rats and study functional effects within 8 hours of infection. Preliminary indications are strong that this goal can be reached.

Further development of these approaches should open new avenues of investigation for the study of physiology in a range of cells and tissues.

Reilly, A.M., Petrou, S., Panchal, R. & Williams, D.A. (2000) Journal of Molecular and Cellular Cardiology, (Submitted for publication).

HYBRID SKELETAL MUSCLE FIBRE - A RARE OR COMMON PHENOMENON?

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In its current use, the term 'hybrid muscle fibre' describes a muscle cell that co-expresses a multitude of myosin heavy chain (MHC) isoforms. Hybrid muscle fibres are also referred to as 'polymorphic fibres' or as 'MHC hybrids'. Early evidence of MHC polymorphism in single muscle fibres can be found in a histochemical study that identified human muscle fibres displaying intermediate staining patterns of myofibrillar ATPase (Staron *et al.*, 1983).

Due to ongoing refinement/development of microanalytical methods for studying proteins and mRNA in single muscle fibre preparations and to imaginative combination of molecular and physiological techniques, the existence of hybrid fibres has been fully confirmed and new knowledge is being sought/produced, at a very fast rate, regarding the number of MHC isoforms, the extent of muscle fibre polymorphism and the physiological, clinical and experimental significance of hybrid muscle fibres. The methods most commonly used for analysis of hybrid fibres include sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, *in situ* hybridization and reverse transcription-polymerase chain reaction (RT-PCR) (Pette *et al.*, 1999).

This review examines data obtained in several laboratories (including our own), which indicate that (i) MHC hybrids occur not only in mammalian, but also in amphibian muscles, (ii) MHC hybrids are found in non-transforming muscles (muscles at 'steady-state') as well as in muscles undergoing phenotypical adaptation to changes in the hormonal milieu, innervation pattern and mechanical demands, (iii) the proportion of MHC hybrids is muscle-specific and, for a given muscle, anatomical region-specific (as it has been shown for laryngeal muscles) or even fibre region-specific (as it has been shown for anterior tibialis muscle fibres of the frog), (iv) with some exceptions, MHC isoform co-expression in single fibres follows a definite pattern, and (v) knowledge of the MHC isoform composition of a single fibre allows only limited prediction of its contractile or metabolic characteristics. The review will also consider evidence of other forms of single fibre polymorphism, in which there is no correlation between MHC isoform and MLC isoform expression, between MHC mRNA and MHC isoform composition ('mRNA-MHC mismatch') or between MHC isoform composition and parameters of sarcoplasmic reticulum (SR) function.

A survey of the literature covering the last two decades reveals that hybrid fibres were once regarded as a rare phenomenon and discarded from investigations concerned, for example, with the contractile properties of MHC-based fibre types (Danielli-Betto *et al.*, 1990). Now there is compelling evidence to suggest that, in some skeletal muscles (such as rat extensor digitorum longus, EDL) MHC hybrids represent the dominant rather than a minor phenotype and, more importantly, that hybrid fibres could be a valuable model for studying the physiological role of muscle protein isoforms and the regulation of gene expression in a multinucleated cell.

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NH&MRC have been the major supporters of work on hybrid fibres carried out in the Muscle Cell Biochemistry Laboratory at Victoria Institute of Technology.

DOES Ca^{2+} RELEASE FROM INTRACELLULAR Ca^{2+} STORES INFLUENCE THE HEART RATE?

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The heart rate is determined by the firing rate of a small group of specialised pacemaker cells. Early electrophysiological studies established that the spontaneous firing of pacemaker cells was due to a period of spontaneous diastolic depolarisation, known as pacemaker potential, which preceded the action potential. Several membrane currents have been identified as contributing to the pacemaker potential but there is no consensus as to the most important pacemaker current. It has long been recognised that changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) affect the firing rate. Recently it has been suggested that Ca^{2+} release from intracellular Ca^{2+} stores, mainly from sarcoplasmic reticulum (SR), is a major factor that determines the heart rate (reviewed by Terrar & Rigg, 2000)

However, there are several questions that need to be addressed before the role of SR Ca^{2+} release in cardiac pacemaking is established. How much Ca^{2+} can be released from the SR given that structural studies show the SR to be poorly developed in pacemaker cells? Can action potentials be generated without Ca^{2+} release from SR? What is the trigger for SR Ca^{2+} release? What is the membrane current that is directly related to $[Ca^{2+}]_i$ in pacemaker cells? Is the increase of the heart rate by β-adrenergic stimulation also mediated by increased Ca^{2+} release from SR?

Studies in toad sinus venosus pacemaker cells from our laboratory have attempted to answer these questions. We simultaneously measured $[Ca^{2+}]_i$ and action potential /membrane currents in isolated single pacemaker cells. Caffeine has been used to measure Ca^{2+} content in SR. SR Ca^{2+} release was manipulated by using ryanodine or SR Ca^{2+} pump blocker, 2,5-di(tert-butyl)-1,4-hydroquinone (TBQ). Confocal imaging has also been used to study the distribution of Ca^{2+} during spontaneous firing.

Our major findings are: (i) $[Ca^{2+}]_i$ rises from diastolic level (~ 200nM) to systolic level (~650 nM) during the spontaneous action potential. (ii) Ca^{2+} extrusion from cells was able to produce a significant inward Na^+ - Ca^{2+} exchanger current which would contribute to the pacemaker currents (Ju & Allen, 1998). (iii) About 50% of the $[Ca^{2+}]_i$ transient resulted from Ca^{2+} released from SR. (iv) Spontaneous firing generally ceased when the SR was depleted. (v) L-type Ca^{2+} current and reverse mode of Na^+-Ca^{2+} exchange are major sources of Ca^{2+} influx that trigger Ca^{2+} release from SR (Ju & Allen, 2000a). (vi) β-adrenergic stimulation increases heart rate by modulating Ca^{2+} influx and SR Ca^{2+} release rather than directly acting on Na^+-Ca^{2+} exchanger (Ju & Allen, 1999a, b). (vii) Ca^{2+} sparks are present in pacemaker cells and increase in frequency in the late phase of the pacemaker potential (Ju & Allen, 2000b).

These results give new insight into the mechanism of firing of pacemaker cells. Such ideas may also be important for understanding the mechanism of cardiac arrhythmias.

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CLINICAL RESEARCH: INTRODUCTION

Judith A Whitworth, John Curtin School of Medical Research, Canberra

John Ludbrook's distinguished research career has been multi-faceted, from surgery to cardiovascular physiology to biostatistics, and characterised by methodological rigor, whether in the operating theatre, the laboratory or at the computer.

Some of his many contributions will be highlighted and illustrated.

RESEARCH: THE PURSUIT OF THE NEW AND THE TESTING OF THE OLD

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My research career commenced when I was a surgical registrar with John Ludbrook, who was then Professor of Surgery in Adelaide. I was involved with two studies: a) A physiological study in normal controls and patients of the influence of acute sympathetic stimulation on hand blood flow using venous occlusion plethysmography, and b) A clinical study of gastric polyps. I learned several important things from these studies. First, that the physiological study in which we were attempting to produce new and (we hoped) useful knowledge, was what most people think of when we talk of research. Second, that retrospective clinical studies are only really useful to the person undertaking the study, and that they are unlikely to produce new knowledge. And third, and most importantly from my perspective, was that attention to detail, and getting it right, are perhaps the most important attributes a researcher can have. In other words, quality is everything.

The importance of obsessive attention to detail, if quality is to be produced, was reinforced for me when I returned as a Senior Lecturer with John Ludbrook several years later, and joined his program studying carotid baroreceptors in conscious rabbits. With his departure from the Department of Surgery, my research strayed every more widely from its vascular beginnings. My interests began to focus on oesophageal and gastric surgery, and in particular, gastric emptying, which we investigated at both a physiological level in an animal model, and at a clinical level. Disappointingly, because of lack of funding, the animal model project was halted.

However, the development of laparoscopic surgery in the early nineties provided us with an opportunity to test the old methods of surgery prospectively. And evidence based medicine has taught us that research is not only the pursuit of the new, but also the vigorous testing of beliefs already held. And, as always, the principle of attention to detail, quality always uppermost, has stood me in good stead as our department has embraced the era of evidence based medicine.

NEW APPROACHES TO THE UNDERSTANDING OF BARORECEPTOR REFLEXES

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It is appropriate in this symposium reflecting the contributions of Professor John Ludbrook, to acknowledge the impact he has made to our understanding of the role of the baroreceptor reflex in the short term regulation of blood pressure and blood flow. Driven by a need to apply more rigorous and critical approaches to assessing afferent inputs to the central nervous system, John has over the years, helped clarify our understanding of arterial and non-arterial baroreceptor reflex mechanisms. In 1984, he wrote "The development of new methods for describing the capacity of reflexes to control blood pressure in human or experimental hypertension poses less of a problem than the recognition that existing methods have grave deficiencies" (Ludbrook, 1984). His contributions to remedy this situation have been many and include a sigmoidal curve fitting computer program, development of the neck chamber approach and its animal counterpart, the carotid capsule for rabbits, to name a few. Much of this work has profoundly influenced the thinking and direction of the newer participants in this field such as myself. I would therefore like to continue his theme and to briefly review some of the newer twists to the more traditional methodology and an assessment of the increasingly popular "non invasive" approaches.

In assessing baroreceptor reflex gain, the most common approach is to determine baroreflex slope using a linear model. However if the sigmoidal model is used then there is additional information such as the range of the reflex which is an indication of the maximum capacity of the reflex and also the curvature which reflects the amount of blood pressure excursion required to reach these maxima. These two parameters are independently influenced by various physiological events. For example partial de-afferentation leads to reductions in both parameters, while de-efferentation leads to a reduction in only the range parameter. Changes to the central integration of the reflex alters the curvature. A recent development has been the five parameter logistic equation, which has two independent estimates of the curvature, thus allowing for non-symmetrical fitting procedures which significantly improve the curve fit. Newer dynamic methods of baroreflex assessment such as power spectral analysis and the sequence method offer a non-invasive estimate of baroreflex gain simply by measuring blood pressure fluctuations. Studies show that these techniques can be used to accurately estimate the gain under a number of states including hypertension but they cannot be used to calculate either range or curvature. Thus the prediction by John Ludbrook concerning the development of new methods for baroreflex assessment outpacing our ability to fully understand existing methods appears to have been quite true, and I suspect will continue to be so for some time.

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NO DEBATE? ENHANCED TOTAL PERIPHERAL VASCULAR RESPONSIVENESS IN HYPERTENSION ACCORDS WITH THE AMPLIFIER HYPOTHESIS

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Since the early work of Folkow (1982) and his colleagues with the perfused SHR and WKY rat hind limb preparation, it is considered that changes in vascular resistance to constrictor drugs are amplified in SHR because of structural remodelling of the vasculature. A reduced internal radius (r_i) and increased wall thickness (ω) to lumen radius ratio (ω/r_i) can theoretically explain this increase in reactivity in hypertension.

We have published work from the conscious rabbit with bilateral renal cellophane wrap experimental hypertension, confirming that amplified resistance increases to constrictor drugs, and amplified falls in resistance to dilator drugs, are a feature of the hindquarter vasculature (Wright & Angus, 1986). Recently, we extended the observations to the 'total' peripheral circulation in the renal hypertensive rabbit (Wright & Angus, 1999). The more sophisticated technology used in this study such as the Transonic™ flow probe chronically implanted on the aortic root and the chronic indwelling left atrial catheter, owes much to Professor John Ludbrook's research at the Baker Medical Research Institute and Department of Surgery, University of Melbourne. In our study we showed that total peripheral resistance and conductance changes to left atrial infusions of methoxamine and adenosine do indeed accord with the amplifier hypothesis. Moreover, total pharmacological antonomic blockade, neurohumoral block or control conditions gave insights into the role of cardiac and vascular homeostatic mechanisms.

There is some debate as to whether the phenomenon of the vascular resistance amplifier does occur in the total circulation and in all types of hypertension (Izzard *et al.*, 1999; Korner *et al.*, 2000). In this presentation, we will show how integrated whole animal pharmacology and physiology can benefit the analysis of an important fundamental problem in hypertension research.

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CARDIAC AFFERENT AND ENDOGENOUS OPIOID MECHANISMS IN THE HAEMODYNAMIC RESPONSES TO HYPOVOLAEMIA AND HYPERVOLAEMIA

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The haemodynamic response to acute central hypovolaemia consists of two phases. During Phase I, arterial pressure is well maintained in the face of falling cardiac output, by baroreceptor-mediated reflex vasoconstriction and cardio-acceleration. Phase II commences once cardiac output has fallen to a critical level of 50-60% of its resting value, equivalent to loss of about 30% of blood volume. During Phase II, sympathetic vasomotor and cardiac drive fall abruptly, and cardiac vagal drive increases. In man, this response is invariably associated with fainting, and has been termed vasovagal syncope.

Although it had been suggested by others previously, the first conclusive evidence for a role of cardiac afferents in triggering Phase II of the response to hypovolaemia came from the work of Öberg and colleagues (see Thorén, 1979). In a series of experiments in anaesthetized cats, they obtained strong evidence that the bradycardia and sympathoinihibition that occurs during severe haemorrhage or caval occlusion is dependent on paradoxical firing of ventricular vagal afferents. More recent studies in conscious rabbits support this hypothesis, since instillation of procaine into the pericardial sac, or cervical vagotomy can prevent the occurrence of Phase II of the response to acute central hypovolaemia (see Ludbrook & Ventura, 1996).

Phase II of the response to acute central hypovolaemia also appears to depend on a δ_1-opioid receptor mechanism in the brainstem, since in conscious rabbits it can be abolished by 4th ventricular administration of opioid antagonists with high affinity for δ_1-opioid receptors, at doses 100-1000 fold less than those required by the intravenous route (see Evans *et al.*, 1989; Ludbrook & Ventura, 1994).

There is also now good evidence that opioid receptor mechanisms mediate circulatory responses to activation of other cardiac mechanoreceptors. For example, in conscious rabbits intravenous administration of the opioid antagonist naloxone blunts the diuretic and natriuretic responses to plasma volume expansion (Shweta *et al.*, 1999), and the sympathoinhibitory response to cardiac tamponade (Hagiike *et al.* 1999). Both these responses rely heavily on vagal afferent signals from the heart (Badoer *et al.*, 1998; Hagiike *et al.* 1999; Shweta *et al.*, 1999). On the other hand, brainstem opioid receptor mechanisms do not make major contributions to the neural pathways subserving reflexes arising from arterial baroreceptors (Evans *et al.*, 1989) or the cardiac chemoreceptors involved in 'Bezold-Jarisch-like' reflexes (Evans & Ludbrook, 1991).

These data are consistent with the notion that neural pathways mediating reflexes arising from mechanosensitive afferents travelling in the cardiac vagus converge within the brainstem at opioidergic synapses. This hypothesis remains to be rigorously tested.

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NON-OPIOID CENTRAL NERVOUS SYSTEM MECHANISMS IN THE HAEMODYNAMIC RESPONSES TO HYPOVOLAEMIA

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The haemodynamic response to acute central hypovolaemia consists of two phases. During Phase I, cardiac output falls while arterial pressure is maintained by baroreceptor-mediated reflex vasoconstriction and cardio-acceleration. Phase II commences once cardiac output has fallen to a critical level. During Phase II, sympathetic vasomotor and cardiac drive fall abruptly, and cardiac vagal drive increases.

A serotonergic mechanism appears to be involved in the onset of Phase II of the response to simulated haemorrhage as methysergide prevents its onset (Evans *et al.*, 1991). This effect is mediated in the brainstem, since methysergide is at least 10-fold more potent when administered into the fourth ventricle than when given intravenously (Evans *et al.*, 1991). Comparison of the relative potencies of a number of serotonin receptor ligands suggests that stimulation of 5-HT_{1A} receptors could explain the inhibition of Phase II of the response to acute central hypovolaemia in conscious rabbits (Evans *et al.*, 1991; 1993).

Intravenous injection of adrenocorticotrophin (ACTH) fragments induces a potent and sustained reversal of haemorrhagic hypotension in cats and dogs (Bertolini *et al.*, 1989) and ACTH(1-24) has been successful in restoring blood pressure in haemorrhagic shock in humans (Noera *et al.*, 1989; Pinelli *et al.*, 1989). Experiments in conscious rabbits have shown that ACTH(1-24) acts within the brainstem to prevent the decompensatory Phase II of acute central hypovolaemia (Ludbrook & Ventura, 1995). The δ-opioid receptor agonist DPDPE abolishes the protective effect of ACTH (1-24) (Ludbrook & Ventura, 1995) which is consistent with an earlier proposal that there is an ACTH-opioid balance which is upset by haemorrhage (Bertolini *et al.*, 1986).

Nitric oxide is involved in the pathophysiology of haemorrhagic shock as inhibitors of its production improve the survival of rats subjected to experimental haemorrhagic shock (Zingarelli *et al.*, 1992). Central nitric oxide mechanisms appear to be involved since nitric oxide synthase inhibitors are able to inhibit Phase II with a greater potency when administered centrally rather than peripherally (Ventura & Ludbrook, 1995a). These central nitrergic and opioid/ACTH pathways appear to interact with each other since L-arginine can reverse the abolition of Phase II caused by the opioid receptor antagonist naloxone (Ventura & Ludbrook, 1995b).

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NEURAL MECHANISMS IN HYPOVOLAEMIA: STUDIES USING IMMEDIATE EARLY GENE EXPRESSION

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Hypovolaemia , such as that which occurs following an acute haemorrhage, results in a powerful compensatory response, consisting of activation of the sympathetic outflow to the cardiovascular system together with an increase in the levels of circulating hormones such as angiotensin II and vasopressin. The signals that reflexly evoke such responses include inputs from arterial baroreceptors (unloaded by the fall in arterial pressure commonly associated with hypovolaemia) and cardiac receptors (unloaded by the fall in atrial pressure). Several studies using the expression of the immediate early gene c-*fos*, a marker of neuronal activation, have shown that hypovolaemia activates neuronal populations in several regions in the brainstem and hypothalamus, but have not determined the relative contribution of various inputs to the observed pattern of activation. This presentation will review studies from our laboratories which were aimed at determining the contribution of baroreceptor and non-baroreceptor inputs to the pattern of c-*fos* expression in the brainstem and hypothalamus that is evoked by hypovolaemia.

In a recent study (Potts *et al.*, 2000) we determined the pattern of c-*fos* expression in the medulla and hypothalamus induced by central hypovolaemia in both intact and barodenervated conscious rabbits. Central hypovolaemia resulted in significant decreases in right atrial pressure and arterial pressure in both groups of animals. There was also no significant difference in the pattern of c-*fos* expression in the two groups, indicating that this is primarily a consequence of unloading of cardiac receptors, together with an increase in the levels of angiotensin II and vasopressin. In other studies we have determined the pattern of c-*fos* expression in the medulla and hypothalamus that is induced specifically by unloading of arterial baroreceptors (Li & Dampney, 1994; Potts *et al.*, 1997) or to an increase in the level of circulating angiotensin II (Potts *et al.*, 1999).

The collective results from these studies demonstrate that signals arising from arterial baroreceptors and cardiac receptors in both cases activate neurons in several distinct medullary regions (e.g. nucleus of the solitary tract and ventrolateral medulla) and hypothalamic regions (e.g. supraoptic nucleus, paraventricular nucleus, and circumventricular organs). At the same time, the neuronal populations activated by each of these signals are, at least in part, quite separate, even though they may overlap in their anatomical locations. These studies indicate that, in response to hypovolaemia, the pattern of activation of medullary and hypothalamic neurons will depend upon the relative degree of activation of neural and hormonal signals, which in turn depends upon the degree of hypovolaemia that is produced.

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STATISTICS IN PHYSIOLOGY AND PHARMACOLOGY: A SLOW LEARNING CURVE

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This is an autobiographical account of my attempts to understand how best to analyze my results (and, latterly, those of others) statistically. It starts with a χ^2 test, executed with pencil and paper (1951), through *t*-tests executed by way of slide rule (1957), Fisher's exact test executed by a Burroughs electro-mechanical calculator (1965), simple analyses of variance executed by hand-held electronic calculator (1971), through somewhat more complex analyses executed on mainframe and PDP computers (1978), to an increasing variety and complexity of analyses executed with personal computer since the mid-1980s, including clinical studies and clinical trials since 1997. My account includes a confession about the first paper I read before the Society (1965), after which the way I presented my statistical analyses was torn to shreds by a distinguished physiologist and mathematician. Over the past 50 years my understanding and application of statistical analysis has followed a slow learning curve, though I shall argue that statistical maturation is inevitably a slow process.

I address some of the issues that the younger generation of physiological and pharmacological investigators seem not to comprehend. Among these is the matter of making multiple inferences from a single experiment(Ludbrook, 1994). This is an issue that molecular, cellular and tissue biologists have special difficulties in grasping. Another matter is estimating sample size/power in advance of experiments. This has special importance in clinical studies, though a brave attempt to persuade laboratory experimenters of its importance in animal studies (McCance, 1989) appears not to have succeeded. A further matter is the eagerness with which investigators embrace the ready availability of statistical computer software, without really understanding what they are doing with it. This is not always their fault. There are serious limitations to computer statistical software. These include failure to provide adequate documention and references and, in some cases, flawed algorithms (Bergmann *et al.*, 2000).

To revert from the past to the present, physiological and pharmacological investigators are having a hard time, statistically speaking. Increasingly, journals and grant-giving bodies impose compulsory reviewing by statisticians. Yet at the same time the availability of competent and experienced biostatisticians who could provide reliable advice to biomedical investigators diminishes. Biomedical investigators who attend courses on statistics are consistently disillusioned by their irrelevance; and those who seek help from consulting services complain that many hours of consultation (at $80-100 per hour) result in little more than mutual misunderstanding.

In Australian biomedical research institutes, the true cost of production of each published paper is $100-110 000. The investment of a tiny fraction of this cost in ensuring access to professional biomedical statistical advice would seem to be a prudent investment, not only for the present but in ensuring that a sufficient number of qualified biostatisticians will be available in the future.

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INTEGRATION OR DISINTEGRATION?

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In the coming decades, one of the greatest challenges in medical research will be to successfully integrate knowledge at many levels. Genomics gives us the power to understand the basic blocks from which life operates. But, as we move up the scale of complexity towards a fuller understanding of how whole individuals operate and what causes ill health, there is a rapidly increasing degree of complexity of interactions with environment - physical, social, cultural, psychological, time and so on. If physiologists grasp the opportunities that this brings, we can play a central role in helping to understand these complex relationships. To do this we will need to understand functional genomics, to incorporate bioinformatics in our approaches and to act as links between genetic reductionism and population health approaches.

EXERCISE AND SKELETAL MUSCLE GENE EXPRESSION

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Mature skeletal muscle is a remarkably adaptive tissue. One of the most powerful stimuli for inducing skeletal muscle cellular re-organisation is exercise training. These adaptations in response to exercise require the coordinated activation of many complex molecular events, resulting in changes in the mRNA abundance of many thousands of genes. New technologies, particular high throughput gene scanning tools (including gene-chips) will provide dramatic insights into the complex patterns of gene expression necessary for the development of particular phenotypes. However, there are several major considerations that must be taken into account in attempting to understand the relationship between levels of differing mRNA and cellular responses. Many studies have failed to demonstrate an obvious relationship between mRNA abundance and that of the encoded protein. This is particularly true of correlative studies examining gross determinants of gene activation (ie gender, adiposity or habitual activity) with the mRNA abundance of target genes. These findings are not surprising given the transitory nature of gene transcription, with both the timing and duration of the alterations in gene transcription highly variable between differing genes. This is particularly true for a complex stimuli, like muscle contraction, in which multiple transcriptional pathways are activated. Gene transcription may be activated within seconds of contraction initiation, through to hours after the cessation of exercise. Similarly, the maintenance of increased cellular mRNA is dependent upon the combined influences of transcription (synthesis) and degradation (half-life). This is yet to receive systematic attention when examining the gene responses to exercise interventions.

A pronounced adaptive response to high-intensity or weight-bearing exercise interventions is muscle hypertrophy. The increased mass of active muscle groups is achieved by an increase in the volume of individual myofibers. This is dependent upon the fusion of new nuclei, as a constant ratio of nuclei to cytoplasmic volume is maintained. The additional nuclei are supplied through the activation of mononucleated ‘stem-cell like’ satellite cells. Discrete members of the myoD family of transcription factors are required for the proliferation and maturation of satellite cells. Considerable progress has been made in elucidating the signaling pathways responsible for the activation of satellite cells, highlighting the pivotal role of locally synthesised insulin-like growth factors (IGFs).

In contrast, endurance exercise typically results in a shift in myofibrillar components towards an increased abundance of slow isoform proteins, together with up-regulated mitochondrial and oxidative metabolism enzyme levels. Induction of the slow-isoform gene program is dependent upon calcium-dependent activation of the calcineurin – NFAT pathway. Induction of genes encoding slow isoform proteins are responsive only to repeated exercise bouts, reflecting the requirement to significantly up-regulate energetic demands on the muscle and the conservative molecular responses to structure proteins with extended half-lives. Increased mitochondrial oxidative capacity can be induced by as little as 5 days of endurance training, suggesting a more rapid coordinated activation of mitochondrial genes, encoded on both mitochondrial and nuclear DNA. Interestingly, there is a rapid adaptive up-regulation of genes encoding proteins involved in aspects of energy homeostasis metabolism. For example, our laboratory has demonstrated enhanced expression of genes involved in the glucose oxidative pathway within one hour of moderate endurance exercise. Yet this finding is not universal, with adaptive changes in lipid oxidative genes requiring repeated bouts of exercise training.

REGULATION OF SKELETAL MUSCLE GLUCOSE UPTAKE DURING EXERCISE

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Skeletal muscle glucose uptake is increased by exercise. The increased uptake is a function of both an increase in muscle glucose extraction and in muscle blood flow. The latter is quantitatively the most important factor since muscle blood flow may increase up to 20 fold whereas glucose extraction only increases 2-3-(4) fold (Richter, 1996). The contraction-induced increase in glucose transport is in large measure due to translocation of GLUT4 to the surface membrane as indicated by the almost total absence of contraction-induced muscle glucose transport in GLUT4 knockout mice (Zisman *et al.*, 2000). GLUT4 is expressed in a muscle fiber type specific manner. Yet, in human quadriceps muscle the difference in GLUT4 expression between type I and type II fibers is only in the order of 20% (Daugaard *et al.*, 2000) which is much less than in rat skeletal muscle. Physical training increases muscle GLUT4 expression but decreases muscle glucose utilization during submaximal exercise. We have recently shown that this is due to a decreased translocation of GLUT4 to the surface membrane in trained muscle (Richter *et al.*, 1998). However, if exercise is carried out at a high relative work load (80-100% of peak VO_{2max}) glucose uptake is in fact increased in trained muscle. Furthermore, the increase seems to be dependent on the training-induced increase in muscle GLUT4 protein expression (Kristiansen *et al.*, 2000).Contraction-induced muscle glucose transport and GLUT4 translocation is dependent on the pre-contraction muscle glycogen concentration, however only in fast-twitch fibers (Derave *et al.*, 1999). The exact mechanism behind this effect of glycogen is unknown but may be related to glycogen dependent activation of 5`AMP-kinase. However, recently we have described that in contracting rat slow-twitch muscle activation of AMPK is dissociated from activation of glucose transport (Derave *et al.*, 2000). AMPK is activated in an intensity and isoform specific manner in human skeletal muscle during exercise (Wojtaszewski *et al.*, 2000). Its role in regulation of muscle glucose uptake is, however, still unclear.

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GENDER APSECTS OF LIPID METABOLISM DURING EXERCISE

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Conflicting results appear in the literature regarding gender differences in substrate utilisation during exercise. Thus, some studies, based on RER determinations, have revealed that females utilise lipids to a greater extent during exercise at the same relative workload than males, whereas other studies have revealed similar contribution of lipids and carbohydrates to the oxidative metabolism during exercise. One reason for this discrepancy could be ascribed to different experimental protocols. Furthermore, studies vary considerably regarding the degree of matching, the training status of the subjects, the menstrual status of the females, phase of menstrual cycle where testing of the females occurred, the nutritional status of the subjects and type, duration and intensity of exercise performed.
Different lipid sources, albumin-bound fatty acids (FA), VLD lipoprotein-triacylglycerol and triacylglycerol within the skeletal muscle, could contribute to the overall lipid oxidation during exercise. Possible gender differences in the relative contribution from these three lipid sources is not well known.

Recently we studied well-controlled, well-matched untrained and trained females and males exercised on a bicycle ergometer at the same relative submaximal work load (60 % peak VO_2) for 90 min. The femoral artery and vein were catheterized and a muscle biopsy was obtained before and after exercise. Females were studied in the mid-follicular phase. We found similar contribution from carbohydrates and lipids to oxidative metabolism, evaluated by means of RER. Despite similar arterial FA concentrations during exercise we observed that plasma FA net uptake across the exercising leg was higher in the trained males than in the untrained. In both the untrained and the trained females plasma net uptake of FA was similar and not different from that in the untrained males.

The content of intramuscular located triacylglycerol (IMTG) at rest was significantly larger in the females than in the males, irrespective of training status, even though both females and males followed a similar carbohydrate-rich diet during the 8 days preceding the experimental trial.

During exercise utilisation of IMTG was not observed in the male subjects. This supports our earlier findings and is in accordance with other studies but in contrast to some. On the contrary, irrespective of training status, the females utilised significant amounts of intramuscularly located triacylglycerols during submaximal exercise at the same relative workload as the males.

The mechanism behind this gender difference is unknown. A possible underlying mechanism could be a higher concentration of TG-lipase responsible for the lipolysis of IMTG and/or a higher catecholamine sensitivity of this TG-lipase in females compared to males.

LARGE ARTERY MECHANICAL ADAPTATIONS TO EXERCISE TRAINING: IMPLICATIONS FOR CARDIOVASCULAR RISK

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The stiffness or compliance of the proximal aorta is the principal determinant of central pulse pressure with stiffer large arteries giving rise to pulse pressure elevation through higher systolic and lower diastolic pressure. Systolic pressure is an important determinant of cardiac work while diastolic pressure is the driving force for coronary perfusion (Watanabe *et al.*, 1993). Aortic stiffness is thus a key factor in the relationship between myocardial blood supply and demand with important consequences for myocardial work capacity. Such effects would be expected to impact on both exercise capacity and cardiovascular risk. In support of a role for arterial properties in exercise performance, aerobically trained athletes (30-59 years) have lower arterial stiffness than their sedentary counterparts and VO_2max correlates inversely with large artery stiffness (r=0.44; P<0.001) (Kingwell *et al.*, 1995). Furthermore in older subjects (57-80 years) time to exhaustion on treadmill testing correlated positively with arterial compliance (r=0.42; P=0.005) (Cameron *et al.*, 1999). While in this group exercise was not limited by myocardial ischaemia, in patients with any given degree of coronary artery disease, those with stiffer proximal arteries had a lower exercise induced ischaemic threshold (Kingwell *et al.*, 2000). The extent to which 1) a predisposition to a more compliant arterial circulation and lower pulse pressure permits greater exercise/athletic performance or 2) physical training increases large artery compliance and exercise capacity in parallel, cannot be determined by these data.

Since a more compliant proximal circulation may increase exercise performance and perhaps represent an important therapeutic target, it is important to understand the effects of acute and chronic aerobic exercise on large artery stiffness. A single 30 minute cycling bout at 65% VO_2max increased arterial compliance by 40% 30 minutes post exercise, but values returned to resting levels by 60 minutes post exercise (Kingwell *et al.*, 1997a). After training 3 times per week for 4 weeks using the same protocol resting arterial compliance (24 hours after the last exercise bout) was elevated by ~30% (Cameron & Dart, 1994). Since compliance is inversely related to mean distending pressure it is important to determine whether this training effect was simply a result of reduced mean arterial pressure. To this end rats were trained using the spontaneous exercise model from 4 to 20 weeks of age and aortic cross-sectional compliance examined in organ baths (Kingwell *et al.*, 1997b, 1998). The slope of the diameter-tension curve was higher in trained rats indicating that training induced structural aortic adaptations, suggesting that the findings in humans were independent of changes in mean arterial pressure. While aerobic training is effective in elevating compliance in young healthy subjects, recent work suggests that patients with isolated systolic hypertension may have irreversible stiffening which cannot be improved through moderate training.

Finally the abrupt and large pressure elevations associated with muscular strength training result in left ventricular hypertrophy and would be also expected to impact on the structure and function of the aorta. In a cross-sectional analysis, exclusively high resistance, strength trained athletes had stiffer large arteries with an associated elevation in both brachial and carotid pulse pressure (Bertovic, 1999). The clinical implications of these findings with regard to cardiovascular risk are currently unknown.

In conclusion large artery stiffness is an important modulator of the myocardial blood supply and demand equation which has significant ramifications for athletic performance and for ischaemic threshold in coronary disease patients. Moderate aerobic training but not high resistance strength training reduces large artery stiffness in young individuals while older subjects with established isolated systolic hypertension are resistant to such adaptation.

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ACUTE AND CHRONIC RESPONSES TO INTENSIFIED TRAINING IN HUMANS: CONSEQUENCES FOR PERFORMANCE

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Successful athletic performance depends on an individuals capacity to sustain the highest average power output (W) or speed (m min^{-1}) for the duration of an event. This, in turn, depends on the rate and efficiency at which chemical energy (from endogenous and exogenous carbohydrate [CHO] and fat) can be converted into mechanical energy for muscular contraction. Training for performance enhancement should aim to induce multiple physiological and metabolic adaptations that i) enable an individual to increase the rate of energy production from both aerobic and oxygen-independent pathways, ii) maintain tighter metabolic control (ie. match ATP production with ATP hydrolysis), iii) minimise cellular disturbances, iv) increase economy of movement and v) improve the working muscles resistance to fatigue during exercise. We have undertaken a series of related but independent investigations on highly-trained subjects (maximal oxygen uptake [VO_{2max}] ~5.0 L min^{-1}) to determine the effects of an acute (single bout) and chronic (2 sessions.wk^{-1} for 6 wk) intensified training on selected skeletal muscle adaptations. Subjects, who are typically cycling ~400 km wk^{-1}, replace a portion (10-15%) of their aerobic training with laboratory supervised high-intensity interval workouts. Each intensified training session consists of 8-10 repetitions at ~85% of VO_{2max} (325-350 W) with 1 min recovery. The metabolic demands of these sessions are high with oxygen uptake values ~4.25 L min^{-1} and rates of ventilation >100 L min^{-1}. There is a reliance on CHO-based fuels for energy production: rates of CHO oxidation are ~340 μmol kg^{-1} min^{-1} and muscle glycogen concentration falls ~50% after a single session. Adenosine 5'-monophosphate-activated protein kinase (AMPK) α_2 (but not AMPKα_1) activity increases significantly in response to an acute bout of intense exercise. There is also a direct effect of a single intensified training session on phosphorylation of the nuclear protein histone H3, which may be involved in chromatin remodelling and subsequent gene expression. Chronic intensified training does not appear to increase the activity of several oxidative or glycolytic enzymes (ie. citrate synthase, 3-hydroxyacyl CoA dehydrogenase, phosphofructokinase, hexokinase). However, there are significant increases in skeletal muscle buffering capacity after chronic training which are related to changes in performance power output. Furthermore, there are decreases in plasma lactate concentration and the rate of CHO oxidation at the same absolute (pre-training) work rate. These adaptations enable previously well-trained endurance athletes to better resist fatigue (ie. sustain a higher power output or speed) after intensified training.

HETEROGENEITY IN THE DISTRIBUTION OF VASCULAR GAP JUNCTIONS AND CONNEXINS: IMPLICATIONS FOR FUNCTION

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Gap junctions permit the transfer of electrical and chemical information between adjacent cells in a wide variety of tissues. In vertebrates, they are comprised of members of a family of membrane proteins called connexins (Cxs), only three of which (Cxs 37, 40 and 43) have been identified in blood vessels (Christ *et al.*, 1996). While all three have been found between adjacent endothelial cells, the situation in the medial layer is less clear, with Cx43 being considered the predominant Cx expressed in vascular smooth muscle. In addition, studies demonstrating true gap junctions connecting the endothelial cells with the inner layer of smooth muscle cells are rare (Spagnoli *et al.*, 1982; Aydin *et al.*, 1991) and no connexins have yet been identified at these myoendothelial gap junctions.

Our studies in the rat have shown that Cx43 is abundantly expressed at the mRNA level in large elastic arteries, such as the aorta, with Cx43 protein being found in both endothelial and smooth muscle layers. By comparison, in muscular arteries, mRNA and protein expression of Cx43 is significantly less, with Cx43 protein not being found in the muscle cell layers with light microscopy. Furthermore, Cx43 expression is variable in the endothelial cell layer in different muscular arteries. In the rat tail artery, for example, both mRNA and protein expression of Cx43 is significantly decreased after the endothelial cell layer has been removed, suggesting that this Cx does not contribute to cell to cell coupling in the smooth muscle layers. At the ultrastructural level, small pentalaminar gap junctions can be found between the processes of adjacent smooth muscle cells.

Serial section electron microscopic studies have shown that pentalaminar myoendothelial gap junctions can be found in muscular arteries of the rat, such as the mesenteric and tail arteries, but that they are often less than 100 nm in size. Furthermore, the incidence of these structures varies both between vascular beds and within the same vascular bed. In the mesenteric vascular bed, the number of myoendothelial gap junctions increased as vessel size decreased. In the same vessels, the role of endothelium-derived hyperpolarising factor (EDHF) in vasodilatory responses to acetylcholine increased, relative to the role of nitric oxide, as vessel size decreased. Furthermore, a significant proportion of the non-nitric oxide mediated responses could be prevented after application of the gap junction uncoupling agent, 18α-glycyrrhetinic acid.

In summary, heterogeneity exists within the vascular system with regard to the distribution of gap junctions and their constituent Cxs. Such variation will have important consequences for coordination and propagation of vascular responses. While Cx43 is widely expressed in both endothelial and smooth muscle cell layers of large conduit arteries, it appears not to be an important component of the medial layer of the smaller muscular arteries. Connections between the two different cellular layers of blood vessels, through myoendothelial gap junctions, also vary in their incidence in different vascular beds. The correlation between the incidence of these structures and the role of EDHF, relative to nitric oxide, in vasodilatory responses suggests that they play an important role in the action of EDHF in both normal and pathological states.

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CELLULAR COUPLING AND CONDUCTED VASOMOTOR RESPONSES

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It has been known for many decades (Krogh *et al.*, 1922) that constriction or dilation of small blood vessels initiated at one point would spread for considerable distances through the vascular tree. This phenomenon is not mediated by nerves or humoral factors carried in the blood and Segal and Duling (1986) suggested that membrane potential changes in the vascular smooth muscle were responsible. When the role of the endothelium in mediating arteriolar dilation was established the endothelium became the prime candidate for conduction, as coupling between endothelial cells was easy to demonstrate by transfer of dyes between cells. However, when it was found that endothelium was sometimes electrically coupled to the muscle it became harder to interpret some of the experimental data.

Our calculations of the spread of membrane potential changes in branched two-layered vessels indicated that membrane potential changes would be expected to spread better in the smooth muscle than the endothelial layer, because the endothelial layer is so thin. From the experimental point of view the most easily observable effect of removing the endothelium would be an increase in input resistance of the tissue. This was confirmed for arterioles of the guinea-pig small intestine, in a project in collaboration with Dr N. Kotecha and Dr S.E. Luff of Monash University.

Several groups have published data on the spread of dilation in a variety of vascular beds, and in general the distance over which dilations spreads seems to be greater than would be expected for purely passive spread of a hyperpolarization. There is usually insufficient data on the electrical properties of vessels to make an exact prediction of spread to compare with experimental data, but it is possible to develop qualitative criteria to decide whether spread is passive or not. We have measured the spread of hyperpolarization in response to locally applied acetylcholine in arterioles of the hamster cheek pouch (in collaboration with Prof S.S. Segal at Yale University), and conclude that the hyperpolarization is spreading further than can be accounted for by passive electrical properties alone.

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EXERCISE IS GOOD FOR THE ARTERIES – TESTING THE DULL HYPOTHESIS

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Doing acute exercise is exquisite control of the circulation with local metabolic, reflex and later hormonal contributions which allow matching of the needs of working skeletal muscle with the maintenance of homeostasis of the remainder of the body. John Ludbrook has made great contributions in this field bringing an integrative approach, novel techniques and analysis. My colleagues and I have been stimulated by this work in pursuing an overlapping series of projects designed to examine whether the long term effects of regular exercise are a result of the acute changes and how they might be beneficial to cardiovascular health.

Much of the conventional wisdom on the benefits of exercise arises from epidemiology which shows that fitter members of the community are less likely to develop hypertension and have lower all cause mortality rates. However these shed little light on the mechanisms involved and it is difficult if not impossible to tease out the direct effects of regular exercise from other associated lifestyle factors. In well controlled intervention studies regular aerobic exercise, lowered blood pressure, increased insulin sensitivity, improved lipid profile, reduced sympathetic activity and increased arterial compliance. There appears to be a window of optimum frequency and intensity of exercise to achieve these benefits but this differs for different physiological variables.

Compared to the sedentary state trained subjects were found to have enhancement of the sympathetic component of the baroreceptor heart rate reflex. They also had reduced overall noradrenaline spillover and the majority of this was accounted for by reduction in the renal bed. Examination of the time course showed that the autonomic changes occurred some weeks after an initial fall in blood pressure and at a time when increased cardiopulmonary volumes and ventricular dilatation could be seen.

Arterial compliance increased with regular exercise both in the short and longer term. There was a close relationship between the change in arterial compliance both within and between subjects and altered gain in the baroreceptor heart rate reflex. This led to an hypothesis that the reflex responses to exercise training were related to altered afferent input from arterial baroreceptors and cardiopulmonary receptors following structural remodelling. Mathematical analysis of the influence of altered carotid artery compliance on baroreceptor firing rates was consistent with this hypothesis.

Although the reflex changes associated with regular exercise are important the time course and other associations suggest that they are secondary to earlier changes at the vascular level.

In resistance vessels there is increased nitric oxide mediated vasodilatation after training. Once again this has a complex time course with alterations in basal NO mediated vasodilatation following a different time course to those in stimulated release by acetylcholine and other agonists. In exercising skeletal muscle nitric oxide has metabolic effects that are at least as important as those on haemodynamics.

The biological responses to regular exercise are multiple, complex, and explicable to a large degree by changes in the major control mechanisms and interactions between them.

PREPARATION OF ABSTRACTS OF COMMUNICATIONS AND DEMONSTRATIONS TO THE AUSTRALIAN PHYSIOLOGICAL AND PHARMACOLOGICAL SOCIETY

1. McCance[*]*, formerly of the Department of Physiology, Monash University, Clayton, Victoria 3168, Australia.*

Material should be submitted on a floppy disk that can be read by an IBM-compatible computer, OR by E-mail to ianmcc@netspace.net.au. The number of words should not exceed 600. The format should conform with the style of abstracts in recent Issues of the *Proceedings*. This abstract is an example, except for its use of headings.

Title. The title should be in grammatical English without a full stop.

Name(s) and Institute address of author(s). The name of the author who will present the communication should be listed first **or** underlined. The address should identify where the work was done. Employ an asterisk in conjunction with a footnote to give an author's address if it differs from where the work was done. **Where no author is a Society member**, the introducing member must be identified in parentheses immediately following the address i.e. *(Introduced by .....)*

Text. The text should contain enough detail to be self-explanatory. If abbreviations are used they must be defined at first appearance (**but not in the title**). S.I. units should be used as recommended by Baron (1988). For statistical notation see McCance (2000).

Citations. Use the Harvard system as employed by the Journal of Physiology, except that the ampersand (&) should appear only in references and bracketed citations, not in the text. Work by three or more authors may be cited using the form Aitkin *et al.* (1993) (e.g.) unless it will lead to confusion.

References. The cited works must be listed after the text, alphabetically by the authors' surnames, then chronologically if necessary. The reference must include all authors' names, year of publication, full title of journal (e.g., Journal of Physiology, not J. Physiol.), volume number and first and last pages of the article. For a book, include the title, editor, edition if applicable, specific page references if applicable, city of publication and publisher (see, e.g., Baron, 1988).

Footnotes. An example of a footnote is shown below. A line will appear between the references and the footnote(s).

Figure. Only one figure (line drawing or half tone) is permitted. It should bear no title or legend and be unnumbered. Its location should be *within* the text (not before or after), be appropriately described in the text and referred to as "the Figure". Lettering should be approximately 12 pt.

Table. Only one table is permitted. It should be typed in the appropriate position in the text and ruled with 0.5 mm black lines. It should have no number title or legend and be referred to as "the Table".

Animal Experiments. Note that Domestic rule 11(5) (see Members Handbook) states that "All abstracts that deal with animal experimentation *in vivo* should include the names, doses (where applicable) and modes of administration of all anaesthetic, tranquilizing and muscle relaxant drugs employed". Users of *in vitro* materials should make it clear that tissue was removed from anaesthetized or dead animals. The inclusion of such information is for the protection of authors and the Society.

Baron, D.N. (1988) In: *Units, Symbols and Abbreviations*, ed. Baron, D.N. pp. 1-64.
London: The Royal Society of Medicine.

McCance, I. (2000) Proceedings of the Australian Physiological and Pharmacological Society, 31(2), 137P.

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STANDARDIZATION OF STATISTICAL NOTATION IN THE PROCEEDINGS OF THE AUSTRALIAN PHYSIOLOGICAL AND PHARMACOLOGICAL SOCIETY

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The book by Ellis (1972), previously recommended as a guide for symbols and abbreviations, is superseded by Baron (1988), q.v.

The entries under "standard error", however, still suggest that some clarification is required before this work can be used to standardize statistical notation for the *"Proceedings"*. The guide-lines that follow are intended to provide this clarification. In all respects other than in reference to the standard error the abbreviations recommended here are those of Baron (1988).

Abbreviation for number of observations. Use lower case (italic) *n*.

Abbreviationfor probability. Upper case (italic) *P* should be used, rather than *p* (Pressure) or p (pico-).

Standard deviation and standard error. Authors should cite standard deviations when they are concerned to describe the variability of individual values about their mean. In such cases the data are likely to be descriptive rather than strictly experimental. Baron (1988) suggests SD or *s*: we prefer to use SD (N.B. A *change* - this differs from previously recommended s.d. (Ellis, 1972)).

Authors should cite standard errors when they are concerned to indicate the precision, as an estimate, of a particular statistic such as a mean. The preferred abbreviation is SE (*change,* no longer s.e.) in all cases where it is clear to which statistic the SE is referred, but (in accordance with the notation used in *Clinical and Experimental Pharmacology and Physiology*) authors may use SEM for the SE of a mean. When quoting a SE it is preferable to include *n*, the number of observations on which the SE is based (see below).

The use of ± . It is normal practice, in the *"Proceedings"* and elsewhere, to use ± to introduce a SE. In the *"Proceedings"* we will disregard the ruling against this usage in Baron (1988). This matter is discussed by Bliss (1967), who agrees that "in the recent literature" a term following a ± sign is unambiguously a SE.

Inappropriate(?) SE. Authors should consider seriously their objective for including SE when these include variability that is not involved in the statistical test of significance used in the study, for example, the SE of means when the analysis uses a paired "*t*" test. In such cases it may be preferable to quote the SE of the mean difference as well as or instead of the individual SE.

Improper SE. Authors should remember that the precision of a statistic or a comparison between statistics will usually involve variability "between animals (experimental units)" . A value of *n* that is inflated by the inclusion of multiple observations on the same animal (experimental unit) must not be used as the basis for calculating the SE, or for carrying out tests of significance, where the generalization is between animals.

Baron, D.N. (1988) In: *Units, Symbols and Abbreviations,* ed. Baron, D.N., pp. 1-64.
London: The Royal Society of Medicine.

Bliss, C.I. (1967) *Statistics in Biology,* p. 137. New York: McGraw-Hill.

Ellis, G. (1972) In: *Units, Symbols and Abbreviations,* ed. Ellis, G. Revised edition, pp. 1-36.
London: The Royal Society of Medicine.

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UNITS, SYMBOLS AND ABBREVIATIONS IN THE *PROCEEDINGS*

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The previous abstracts make reference to Baron (1988) as the source of information for units, symbols, abbreviations and conventions that should be used in the "*Proceedings"*. To simplify the authors' tasks, some of this information is abstracted here.

Units: SI units should be used. Baron (1988) lists SI Base Units (e.g., kilogram, (kg)), Prefixes for SI Units (e.g., milli (10^{-3}), micro (10^{-6}), nano (10^{-9})), SI derived units with special names (e.g., newton, N, = kg m s^{-2}), and Non-SI units accepted for general use (e.g., litre, l *or* L (see footnote to Table), = 1×10^{-3} $m^{3.}$

Derived units should be written sequentially, e.g., ml kg^{-1} min^{-1} as in Regan *et al.* (1994), with no full stop between. Where only two terms are involved, the solidus may be used, e.g., mol/l *or* mol l^{-1}.

Symbols and Abbreviations: Abbreviations should be defined in the text of the abstract, not in the title.

Times: s, min, h, d; prefer not to abbreviate week, month, year.
Statistics: $\bar{x}$, SD, SE (see McCance, 2000), *P, r, t,* χ^2. Always indicate d.f. where appropriate.
Routes of administration: i.v., i.p., i.m., i.c.v.

ampere	A	diameter	diam.	logarithm to base e	$\log_e$ *or* ln
anterior	ant.	editor(s)	ed.	maximum	max.
calculated	calc.	experiment	expt.	minimum	min.
compare	cf.	extracellular fluid	e.c.f.	page/pages	p./pp.
compliance (resp.)	C	similarly, i.c.f.,p.c.v. *or* PCV, r.b.c. *or* RBC, r.m.s.,w.b.c. *or* WBC		relative humidity	r.h.
counts per minute	ct/min	haemoglobin	Hb	temperature	temp.
concentration of substance shown as formula	[ ]	increment	Δ	revolutions per minute	rev/min
cycles per second	Hz	litre	l *or* L‡	volume	vol.

‡*Note:* Baron allows l, L as alternatives. Authors should be consistent.

Approximation: use approx. (or ≈) for measurements. The Latin word circa (c *or ca*) should be reserved for dates.

Baron, D.N. (1988) In: *Units, Symbols and Abbreviations*, ed. Baron, D.N., pp. 1-64. London: The Royal Society of Medicine.

McCance, I. (2000) Proceedings of the Australian Physiological and Pharmacological Society 31(2), 137P.

Regan, J.M., Macfarlane, D.J. & Taylor, N.A.S. (1994) Proceedings of the Australian Physiological and Pharmacological Society, 25(1), 3P.

APPS STATEMENT ON EXPERIMENTS IN WHICH NEUROMUSCULAR BLOCKING DRUGS ARE TO BE USED

1. The animal must be fully anaesthetized throughout the experiment. Analgesia alone is unacceptable.

2. The adequacy of the anaesthesia must be monitored throughout the experiment. **Indicators used must be such as to satisfy an expert referee that the monitoring procedure is sufficient.**

3. An account of an experiment when such paralysants were used must include the name of the paralysant(s) and the anaesthetic(s) and the doses used. It must also state the measures taken to ensure adequate anaesthesia.

NOTES:

(A) Criteria For Ensuring Adequate Anaesthesia

The use of paralysants precludes the use of simple criteria such as the strength of the corneal blink reflex, the character of the breathing and the flexor withdrawal reflex on pinching the paw or achilles tendon.

The variables recommended to be monitored include both autonomic and CNS indicators. Monitoring must be continuous or, if intermittent, frequent.

- **Autonomic indicators:** heart rate, blood pressure, pupil size: together with the effects on these of mild noxious stimuli.

- **CNS indicators:** the EEG

(B) Factors Interfering With Criteria For Anaesthesia

The investigator is responsible for ensuring that the monitoring procedure is meaningful at all times.

For example, **hypocapnia** may synchronize the EEG without causing anaesthesia, **atropine** dissociates the EEG waveform and behaviour in normal alert cats and will interfere with some autonomic indicators, **ketamine** may have atropine-like effects on the EEG.

(C) Use Of Nitrous Oxide

It cannot be assumed that nitrous oxide alone is an adequate anaesthetic. It should be supplemented with another agent: the proportion may be adjusted for the individual animal on the information from the monitoring.

PROCEEDINGS OF THE AUSTRALIAN PHYSIOLOGICAL AND PHARMACOLOGICAL SOCIETY

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