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Free Communications 3: Cardiac Muscle

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Chair: Lea Delbridge

Tolerance of male and female rat papillary muscles to acute metabolic compromise

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The presence of functional oestrogen receptors within cardiac myocytes identifies the heart as a target organ for oestrogen, and raises the possibility that sex hormone effects on the heart may contribute to sex differences in the incidence of heart disease. We have previously reported that, under comparable conditions, the amplitude of the intracellular Ca^{2+} ($[Ca^{2+}]_i$) transient is larger in male as compared to female rat cardiac myocytes (Curl *et al.*, 2001). It has also been suggested that oestrogen can protect the heart against ischemia-reperfusion injury by limiting the associated increase in $[Ca^{2+}]_i$ (Zhai *et al.*, 2000). The aim of the present study was to determine whether there is a sex difference in the decline and recovery of function, and ability to maintain $[Ca^{2+}]_i$ homeostasis, in intact cardiac muscle subjected to acute metabolic compromise.

Left ventricular papillary muscles were dissected from the hearts of adult (300 – 350g) male and female Wistar rats that had been killed by chloroform overdose and decapitation. The muscles were mounted in a chamber located on the stage of an inverted fluorescence microscope to allow for simultaneous recording of force and tissue fluorescence. For monitoring of $[Ca^{2+}]_i$ the muscles were loaded with fura-2 by 3 hr incubation with fura-2/AM. Muscles were equilibrated in HEPES buffered physiological saline solution containing 2.5 mM Ca^{2+}, 10 mM glucose, and aerated with 100% O_2. They were then subjected to 20 min of metabolic inhibition followed by 60 min of recovery. To achieve metabolic inhibition 2 mM NaCN was added to the PSS, and glucose and O_2 were omitted. The temperature was maintained at 30^{o}C and the muscles were stimulated at 0.25 Hz throughout.

Following 20 min of metabolic inhibition developed force had declined to 10.8 ± 1.6 and 12.1 ± 1.8 % of the preceding steady-state control level in male (n = 12) and female (n = 14) papillary muscles respectively. In contrast, the amplitude of the Ca^{2+} transient only decreased to around 75% of the control amplitude in both sexes. Muscles from both sexes recovered with a similar time course, with developed force returning to approximately 90% of control by 60 min. There were also increases in passive force and diastolic $[Ca^{2+}]_i$ during metabolic inhibition, however, the increase in resting force was considerably less than might have been expected from the increase in diastolic $[Ca^{2+}]_i$. Overall, there were no significant differences between the sexes in either the decline in contractile force, increase in resting force, or changes in $[Ca^{2+}]_i$, during 20 min of metabolic inhibition.

Addition of 1×10^{-6} M 17β -oestradiol to the solutions resulted in a slight decrease (around 10%) in contractile force and amplitude of the Ca^{2+} transient in both male and female papillary muscles. The acute presence of oestradiol, however, had no significant effect on the changes in force or $[Ca^{2+}]_i$ that occurred during metabolic inhibition.

The results demonstrate that there are no apparent differences in the tolerance of isolated male and female rat papillary muscles to 20 min of metabolic inhibition. In addition, the acute presence of a high concentration of 17β -oestradiol did not provide any protection against the effects of metabolic inhibition in either sex. In muscles of both sexes there appeared to be some dissociation of force and $[Ca^{2+}]_i$ during metabolic inhibition.

Curl, C.L., Wendt, I.R. & Kotsanas, G. (2001) *Pflügers Archiv,* 441, 709-716.

Zhai, P., Eurell, T.E., Cooke, P.S., Lubahn, D.B. & Gross, D.R. (2000) *American Journal of Physiology,* 278, H1640-H1647.

The rate of reactivation of the cardiac sodium hydrogen exchanger following inhibition with cyanide

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The cardiac sodium hydrogen exchanger (NHE1) has been implicated in ischaemia/reperfusion damage of the heart. Coupled activity of NHE1 and the sodium calcium exchanger (NCX) are thought to cause calcium overload which is responsible for the resulting the contractile dysfunction and tissue necrosis (Allen & Xiao, 2003).

It is believed that the NHE1 is inactivated by some aspect of metabolic inhibition that occurs during ischaemia and reactivated upon reperfusion (Lazdunski *et al.*, 1985; Park *et al.*, 1999). The time course of this reactivation has indirectly been shown to be very rapid. Park *et al.* (1999) demonstrated that $[Na^+]_i$ started to rise within 30 seconds of reperfusion and reached a peak after 5 minutes. In this study we directly examined the rate of proton flux (J_H ($mmol \cdot l^{-1} \cdot min^{-1}$)) via NHE1 in the acid-loaded ventricular myocytes.

Female SD rats (4-6 weeks) were anaesthetised with pentobarbitone. Single ventricular myocytes were isolated from the heart using a combination of enzymatic digestion and mechanical dispersion. Cells were loaded with the pH indicator carboxy- SNARF-1 and perfused with bicarbonate-free HEPES buffered solution, conditions under which NHE1 is the only acid-extruding mechanism. An isolated cell was then exposed to an NH_4Cl (20 mM) prepulse, and the rate of recovery from acidosis was measured (dpH_i/dt). After return of pH_i to the resting level (7.1) the cell was exposed for 10 minutes to a 2mM NaCN followed by a second NH_4Cl prepulse. The rate of recovery from acidosis was then assessed in the presence of NaCN and then upon its removal.

In control conditions J_H was 0.086 ± 0.022 mM/min (mean ± SEM) when J_H values were calculated in the pH_i range 6.84 − 7.0. In the presence of 2mM NaCN, the J_H value decreased to 0.017 ± 0.005 ($P < 0.05$). This data shows that cyanide inhibits the exchanger. Within 30 s of removal of NaCN, the proton flux had increased to 0.0151 ± 0.019 but part of this apparent flux is caused by the metabolic changes associated with removal of cyanide. After the correction for the effects of the removal of NaCN the mean J_H value was 0.108 ± 0.022 whereas the control measured over the same pH range was 0.050 ± 0.017 which is significantly smaller. These data suggest that the NHE1 activity rapidly reactivates after removal of metabolic inhibition and may show a period of enhanced activity.

Allen, D.G. & Xiao, X-H. (2003) *Cardiovascular Research,* **57**, 934-941.

Lazdunski, M, Frelin, C., & Vigne P. (1985). *Journal of Molecular and Cellular Cardiology*, **17**, 1029-1042.

Park, C.O., Xiao, X.H., & Allen, D.G. (1999). *American Journal of Physiology,* **276**, H1581-H1590.

Activity of the cardiac Na^+-H^+ exchanger during ischaemia

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There is dispute about whether the cardiac Na^+-H^+ exchanger (NHE1) remains active during ischaemia (Allen & Xiao, 2003). The conclusion that the NHE1 was active during ischaemia is based on the large intracellular acidosis which would be expected to drive sodium entry on NHE1 and the fact that some NHE1 inhibitors, such as amiloride, reduce the rise of $[Na^+]_i$ during ischaemia (Murphy *et al.,* 1991). However, pH_i shows no recovery during ischaemia and NHE1 inhibitor do not change the pH_i during ischaemia, which suggested that the Na^+-H^+ exchanger was inhibited during ischaemia (Park *et al.*, 1999). We took advantage of a new potent and selective NHE1 inhibitor, zoniporide (Marala *et al.*, 2002) to reassess the activity of NHE1 during ischemia.

Rats were anaesthetised with pentobarbitone and hearts were isolated and stimulated at 5 Hz. Ischaemia was induced by turning perfusion off for 30 minutes. Intracellular sodium ($[Na^+]_i$) was measured with sodium binding benzofuran isophthatale (SBFI).

In control hearts 30 minutes ischaemia increased $[Na^+]_i$ from 7.2 ± 0.2 mM to 17.3 ± 0.7 mM and reperfusion resulted in a large transient increase of $[Na^+]_i$ (peak 31 ± 2.3 mM (*n*=6)). In the presence of zoniporide (1 μM, *n=5*) present throughout ischaemia and reperfusion, ischaemia still caused a similar $[Na^+]_i$ rise to 16.2 ±0.5 mM but the large transient increase of $[Na^+]_i$ on reperfusion was abolished (peak 13.8 ± 2.4 mM). With amiloride (100 μM, *n=4*) treatment, $[Na^+]_i$ was unchanged at the end of ischemia (6.7 ± 0.7 mM) and the increase of $[Na^+]_i$ on reperfusion was abolished (peak 7.4 ± 0.3 mM).

Both zoniporide and amiloride abolished the transient increase of $[Na^+]_i$ on reperfusion, which results from activity of NHE1. However they showed different effects during ischaemia: noly amiloride abolished the $[Na^+]_i$ rise during ischaemia. Amiloride derivatives reduce the persistent Na^+ current (Chattou *et al.*, 2000). Furthermore, the rise of $[Na^+]_i$ during ischaemia is abolished by low concentrations of tetrodotoxin which inhibit the persistent Na^+ current (Xiao & Allen, 1999). Thus we propose that the ability of amiloride to prevent the $[Na^+]_i$ rise during ischaemia arises from inhibition of the persistent Na^+ current. Measurements of the effect of amiloride and zoniporide on persistent Na^+ current are required to confi rm this hypothesis.

Allen, D.G. & Xiao, X-H. (2003) *Cardiovascular Research*, 57, 934-941.
Murphy, E., Perlman, M., London R.E. & Steenbergen C. (1991) *Circulation Research*, 68**,** 1250-1258
Park C.O., Xiao X-H., Allen D.G.(1999) *American Journal of Physiology*, 276, H1581-90
Marala, R.B., Brown J.A., Kong J.X. *et al.* (2002) *European Journal of Pharmacology* 451, 37-41
Chattou S., Coulombe A., Diacono J., Le Grand B., John G. & Feuvray D. (2000) *Journal of Molecular and Cellular Cardiology* 32, 1181-1192.
Xiao, X-H. & Allen, D.G. (1999) *Circulation Research*, 85**,** 723-730

The effect of polyunsaturated fatty acids on cardiac ryanodine and inositol triphosphate receptors

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It is well recognised that the consumption of fish correlates with a reduction in mortality due to cardiovascular disease (Burr *et al.*, 1989). Whole heart studies have identified that dietary fish oil confers protection from cardiac arrhythmias (McLennan, 1993). Many studies have shown that the acute application of the polyunsaturated fatty acids present in fish oil, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to cardiac myocytes significantly reduce the amplitude of the various sarcolemmal ion currents responsible for the cardiac action potential (Xiao *et al.*, 1995; Xiao *et al.*, 1997; Bogdanov *et al.*, 1998). It is believed that this reduction in electrical excitability is the mechanism by which fish oil confers protection from cardiac arrhythmias. However some arrhythmias also arise from abnormal calcium handling by internal stores. Thus it has been suggested that the anti-arrhythmic effects of long chain polyunsaturated fatty acids (PUFAs) may be related to their ability to alter calcium handling in cardiac myocytes (Honen & Saint 2002, O'Neill *et al.*, 2002). Therefore we investigated the effects of EPA and DHA on the kinetics of the cardiac calcium release channels (*i.e.* the ryanodine receptor (RyR) and the inositol triphosphate receptor (IP_3R)).

RyRs and IP_3R isolated from sheep hearts and were incorporated into artificial bilayers formed from a solution of phosphatidylethanolamine and phosphatidylcholine dissolved in either n-decane or n-tetradecane using standard techniques (O'Neill *et al.*, 2003). Cytoplasmic solutions contained 250 mM Cs^+ (230 mM $CsCH_3O_3S$, 20 mM CsCl), 10 mM TES at pH 7.4. Luminal solution contained 50 mM Cs^+ (30 mM $CsCH_3O_3S$, 20 mM CsCl), 10 mM TES and 1 mM $CaCl_2$, pH 7.4.

Concentrations of EPA ranging between 10 and 50 μM, when applied to either the cytosolic or luminal side of the RyR, produced a dose dependent inhibition of RyR open probability with K_I =32 μM and Hill coefficient, n_I = 3.8. This inhibition typically occurred within 30 seconds of application. Inhibition was independent of the n-alkane solvent and whether RyRs were activated by ATP or Ca^{2+}. Like EPA, the cytosolic application of 50 μM DHA also resulted in a reduction in channel open probability.

Like with RyR, the open probability of the IP_3R fell upon the application of 50 μM EPA. IP_3Rs were identified by their activation by IP_3 and inhibition by 10 μM heparin, a reversible IP_3R blocker.

The results suggest that both EPA and DHA affect calcium handling by directly inhibiting RyRs at micromolar concentrations**.** The actions of both EPA and DHA may be mediated via the membrane or by binding to a hydrophobic site on the channel itself. This provides a potential avenue by which PUFAs confer protection from cardiac arrhythmias.

Bers, D.M., Bridge, J.H. & Spitzer, K.W. (1989) *Journal of Physiology,* 417: 537-553.

Bogdanov, K.Y., Spurgeon, H.A., Vinogradova, T.M. & Lakatta, E.G. (1998) *American Journal of Physiology,* 274:H571-579.

Honen, B.N. & Saint, D.A. (2002) *Journal of Nutritional Biochemistry,* 13:322-329.

McLennan, P.L. (1993) *American Journal of Clinical Nutrition,* 57:207-212.

O'Neill, S.C., Perez, M.R., Hammond, K.E., Sheader, E.A. & Negretti, N. (2002) *Journal of Physiology,* 538(1): 179-184.

O'Neill E.R., Sakowska, M.M., Laver D.R. (2003) *Biophysical Journal,* 84: 1-16.

Xiao, Y.F., Kang, J.X., Morgan, J.P. & Leaf, A. (1995) *Proceedings of the National Academy of Sciences of the United States of America,* 92(24):11000-11004.

Xiao, Y.F., Gomez, A.M., Morgan, J.P., Lederer, W.J. & Leaf, A. (1997) *Proceedings of the National Academy of Sciences of the United States of America,* 94(8):4182-4187.

ATP modulates intracellular Ca^{2+} and firing rate through a $P2Y_1$ purinoceptor in cane toad pacemaker cells

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Recent studies on cardiac pacemaker cells have demonstrated that interventions which affect intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) also influence firing rate (Zhou & Lipsius, 1993; Ju & Allen, 1998; Bogdanov *et al.*, 2001). To investigate the involvement of $[Ca^{2+}]_i$ in modulation of heart rate by ATP, we examined the effect of extracellular ATP (10-100 μM) on $[Ca^{2+}]_i$ and spontaneous firing rate in single pacemaker cells isolated from the sinus venosus of cane toads*. In spontaneously firing cells, ATP initially increased peak $[Ca^{2+}]_i$, diastolic $[Ca^{2+}]_i$, and the firing rate. These early effects were followed by a late phase in which the peak $[Ca^{2+}]_i$, diastolic $[Ca^{2+}]_i$ and the firing rate all declined. Previous studies suggested that positive phase was mediated by P2 purinoceptors, activated by ATP, while the negative phase was mediated by P1 purinoceptors, activated by adenosine (Burnstock & Meghji, 1981). To define the subtype of purinoceptors involved we used αβ-methylene ATP, adenosine, and UTP (respectively $P2X_{1,3}$, P1 and $P2Y_{2,4,6}$ selective agonists). However, we found that these agonists caused no significant change in $[Ca^{2+}]_i$ and had little or no effect on firing rate. In contrast the $P2Y_1$ selective agonist 2-MesADP (1 μM) mimicked the biphasic effects of ATP and these effects were inhibited by the non-selective purinoceptor antagonist suramin and by the $P2Y_1$ selective antagonist MSR 2179.

Immunohistochemistry using an anti-$P2Y_1$ antibody demonstrated that $P2Y_1$ receptors were present on the cell surface. To establish the specificity of the antibody we performed Western blotting analysis on the protein extracts from toad tissues including sinus venosus and aorta as well as rat aorta as positive control. The immunoreaction with the $P2Y_1$ antibody resulted in a major band of apparent molecular weight of approximately 57 kDa in all three samples. Thus the $P2Y_1$ antibody recognized a similar molecular weight protein in both amphibian and mammalian tissues as reported by others (Moore *et al.*, 2000).

To investigate the nature of the biphasic response we studied the effect of ATP on Ca^{2+} store content. We found that the effects of ATP were related to the sarcoplasmic reticulum (SR) Ca^{2+} store. After depletion of the SR Ca^{2+} store with caffeine or ryanodine, ATP no longer had any effect on $[Ca^{2+}]_i$ or firing rate. Furthermore, the SR Ca^{2+} store content was decreased during the late phase of 2-MesADP application. The effect of ATP was coupled to phospholipase C (PLC) activity because the PLC inhibitor U-73122 eliminated the effect of ATP.

Our study shows that in toad pacemaker cells, the biphasic effects of ATP on pacemaker activity are mainly through $P2Y_1$ purinoceptors, which are able to modulate Ca^{2+} release from the SR Ca^{2+} store. We propose that inositol 1,4,5-triphosphate generated by PLC facilitates SR Ca^{2+} release causing the early increase in peak $[Ca^{2+}]_i$. The increased Ca^{2+} release partially depletes the SR Ca^{2+} store accounting for the subsequent decline in peak $[Ca^{2+}]_i$.

Bogdanov, K.Y., Vinogradova, T.M., & Lakatta, E.G. (2001) *Circulation Research* 88, 1254-1258.
Burnstock, G. & Meghji, P. (1981) *British Journal of Pharmacology* 73, 879-885.
Ju, Y-K & Allen, D.G. (1998) *Journal of Physiology* 508, 153-166.
Moore, D., Chambers, J., Waldvogel, H., Faull, R., & Emson, P. (2000) *Journal of Comparative Neurology* 421, 347-384.
Zhou, Z. & Lipsius, S.L. (1993) *Journal of Physiology* 466, 263-285.

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Calcium loading properties of sarcoplasmic reticulum from rat ventricular myocardium

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There is a very large range of estimates for SR Ca^{2+} capacity of cardiac muscle in the literature, as reviewed by Bers (2001). For example estimates for ventricular myocardium SR Ca^{2+} capacity may vary by a factor of up to 6 times. In part this range may be attributed to the differences in the functional state of the SR. In this study we tried to emulate the conditions prevalent in "active" and "resting" cardiac muscle by exposing preparations to ATP containing solutions at different concentrations of EGTA and pCa during the skinning procedure. Such conditions are known to either facilitate a prolonged activation (0.1 mmol L^{-1} $EGTA_{Total,}$ pCa 7.1) or prevent any activation (10.0 mmol L^{-1} $EGTA_{Total}$, pCa 9). Kabbara & Stephenson (1997) showed that when Ca^{2+} entry was facilitated during the skinning procedure the SR was loaded with more Ca^{2+} under the same loading conditions compared with skinning when Ca^{2+} entry was prevented. They regarded these responses as being reminiscent of those from "active" cardiac muscle compared with more quiescent or "resting" cardiac muscle.

Right ventricular muscle bundles were homogenised in either highly (10.0 mmol L^{-1} $EGTA_{Total}$, pCa 9) or weakly (0.1 mmol L^{-1} $EGTA_{Total}$, pCa 7.1) calcium buffered skinning solutions to mechanically render the sarcolemma "leaky". The preparations were then subjected to a simple protocol developed to estimate the SR Ca^{2+}content. Briefly, the preparation was immersed into a solution of known $[EGTA]_{Total}$ at a desired pCa for up to 10.0 minutes to allow the SR to equilibrate with calcium. Then the preparation was moved into an identical solution, which also contained 30 mmol L^{-1} caffeine where a caffeine-induced force transient was recorded. This procedure was then repeated without reloading the SR with Ca^{2+}. On second immersion into the caffeine containing solution, there was no SR Ca^{2+} release, and force only increased due to the higher sensitivity of the contractile apparatus for Ca^{2+} in the presence of caffeine. By overlaying the force responses we could subtract the myofibrillar force response component due to caffeine, which allowed measurement of the SR Ca^{2+} released which could then be converted to the amount of Ca^{2+} released by the fibre volume at the peak of the caffeine-induced force response.

The SR Ca^{2+} content estimated from the caffeine-induced force responses for preparations loaded at pCa 7.0, over a range of $[EGTA]_{Total}$ (0.02 to 0.2 mmol L^{-1}) were on average 197 ± 35 (mean ± SEM, n=3) mmol Ca^{2+} L^{-1} cytosol for preparations skinned under weakly buffered conditions for calcium, emulating "active" myocardium. When preparations were skinned in 10 mmol l^{-1} $EGTA_{Total}$ (pCa 9) however, this was reduced to 142 ± 2 (mean ± SEM, n=3) mmol Ca^{2+} L^{-1} cytosol where the preparations were expected to behave more like "resting" muscle. Hence there appeared to be a shift towards higher calcium loading and release by the SR when the preparations have been skinned under conditions analogous to "active" cardiac muscle compared with conditions more like those of the "resting" cardiac muscle.

Bers, D.M. (2001) *E-C Coupling and Cardiac Contractile Force, 2nd Ed.* Kluwer Academic Publishers, Dordrecht, p 179.

Kabbara, A.A. & Stephenson, D.G. (1997) *American Journal of Physiology,*. 273, H1347-H1357.