APPS/PSNZ Meeting - Sydney 2003

Free Communications 2: Ion channels and biophysics

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Chair: Trevor Lewis

NO donors increase persistent sodium current in HEK293 cells transfected with the human cardiac Na^+ channel α-subunit

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Voltage-gated Na^+ channels play an essential role in excitable cells in which they transiently increase Na^+ conductance in response to membrane depolarisation. However, many tissues have a component of Na^+ current that is resistant to inactivation. This persistent Na^+ current (I_{Nap}) plays an important role in generation of rhythmic oscillations in neurons. Pathological changes in these channels are associated with diseases such as ischaemia, cardiac arrhythmias and epilepsy. Nitric oxide (NO), the major endothelium-derived relaxing factor, reduces whole-cell Na^+ current in isolated ventricular myocytes (Ahmmed *et al.*, 2001) but increases I_{Nap} in rat neuronal and cardiac cells (Hammarstrom & Gage, 1999). NO is also a potential endogenous regulator of I_{Nap} under physiological and pathophysiological conditions (Ahern *et al.*, 2000). The target for NO on Na^+ channels is not known.

We have tested the effects of NO on I_{Nap} in HEK 293 cells transiently transfected with the human cardiac Na^+ channel α-subunit. Persistent Na^+ channel activity in inside-out patches was increased ~10 fold after exposure to NO donors, s-nitroso-n-acetyl penicillamine (SNAP) and sodium-nitroprusside (SNP). Our results suggest that the effect of NO on I_{Nap} is caused by NO directly interacting with Na^+ channel α-subunit, or with closely associated protein(s): Na^+ channel β-subunits appear not to be necessary for this effect. The effect of NO on I_{Nap} was inhibited by the sodium channel blocker, lidocaine (50 μM) and by the reducing agent dithiothreitol (DTT, 2 mM).

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Ahmmed, G. U., Xu, Y., Hong Dong, P., Zhang, Z., Eiserich, J. & Chiamvimonvat, N. (2001) *Circulation Research,* **89,** 1005-1013.

Hammarstrom, A. K. M. & Gage, P. W. (1999) *Journal of Physiology,* **520,** 451-461.

Aphidicolin-induced stress pathway in pre-implantation embryos

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The cell cycle is a ubiquitous and complex process that is essential for the proper growth and development of the pre-implantation embryo. There has been increasing evidence that correlates the cell cycle with the activity of ion channels, in particular potassium channels. In a previous study we have shown that aphidicolin-induced G1 cell cycle arrest of pre-implantation embryos results in the constitutive activation of a cell cycle-related potassium channel (Day *et al.*, 1998). The present study was aimed at identifying the various signalling pathways activated upon the administration of aphidicolin using flow cytometry and microarrays and from there, decipher the link between these pathways and potassium channel activity.

Results suggest that aphidicolin-induced cell cycle arrest was due to stimulation of the stress-activated kinase pathway (SAPK) that proceeds via p38MAP kinase. This provides a potential link between the mitogen-activated kinase (MAPK) pathways and the activity of the cell cycle-related potassium channels present in embryos.

Day, M.L., Johnson, M.H. & Cook, D.I. (1998) *EMBO Journal*, **17**: 1952-1960.

Distinct expression of intermediate-conductance calcium-activated potassium (IK) channels in intrinsic primary afferent neurons of the rat gastrointestinal tract

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Intrinsic primary afferent neurons (AH neurons) in the intrinsic ganglia of the small intestine have a broad action potential that is followed by early and late afterhyperpolarising potentials (AHP). Our laboratory has reported electrophysiological evidence consistent with intermediate-conductance calcium-activated potassium channels (IK channels) being responsible for the AHP in enteric primary afferent neurons (Vogalis *et al.*, 1992), however the molecular expression of IK channels has not been reported in these cells. This study was undertaken to investigate whether IK channels are expressed in the enteric nervous system and whether their expression corresponds to those cells known to express AHP currents.

To localise the IK channels, an antibody was generated in rabbits against the N-terminal 15 amino acids of the rat IK channel and immunohistochemistry performed on whole mount preparations of rat gastrointestinal tract. Evidence for specificity of the antibody was shown in Western blots where it was found to recognise a single band of 160kD in HEK 293 cells transfected with IK cDNA plasmid but not in cells transfected with vector alone or vector containing SK2 cDNA, or on blots probed with pre-immune serum.

IK channel immunoreactivity was found in specific nerve cell bodies throughout the gastrointestinal tract, from the esophagus to the rectum. The majority of immunoreactive neurons had Dogiel type II morphology and in the myenteric plexus of the ileum almost all immunoreactive neurons were of this shape. Intrinsic primary afferent neurons in the rat small intestine are Dogiel type II neurons that are immunoreactive for calretinin, and it was found that almost all the IK channel immunoreactive neurons were also calretinin immunoreactive. IK channel immunoreactivity also occurred in calretinin-immunoreactive, Dogiel type II neurons in the caecum. Within immunoreactive cells, the initial segments of the axons contained the highest density of sites, but not axon terminals. No immunoreactivity was found in surrounding muscle or glia.

Molecular evidence for IK expression was determined by RT-PCR analysis using oligonucleotide probes based on the rat IK sequence (Neylon *et al.*, 1990). RT-PCR cloning from a highly enriched myenteric ganglion extract revealed an mRNA sequence that was identical to the IK channel mRNA expressed in other cell types.

It is concluded that IK channels are expressed on specific neurons of the gastrointestinal tract. They are almost exclusively located on cell bodies and proximal parts of axons of intrinsic primary afferent neurons. From functional studies, these IK channels are predicted to control the excitability states of the enteric nervous system.

Neylon, C.B., Lang, R.J., Fu, Y., Bobik, A. & Reinhart, P.H. (1999) *Circulation Research,* 85, e33-e43.
Vogalis, F., Harvey, J.R. & Furness, J.B. (2002) *Journal of Physiology,* 538, 421-33.

Inhibition of human large conductance calcium-activated potassium channels by a fungal toxin

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The aim of this research was to investigate possible receptor/ion channel sites of action of a fungal toxin (designated compound A) that produces ataxia, tremors, and hypersensitivity to external stimuli when injected into mice. Compound A is distinct among neurotoxins in that it has a long duration of action, producing tremors that can last for up to three days rather than only a few hours. It also inhibits electrically stimulated smooth muscle contraction, increases neurotransmitter release, and elevates blood pressure. These effects suggested the disruption of large conductance calcium-activated potassium (BK) channels, as they have important regulatory roles in smooth muscle contraction and in control of neurotransmitter release (Gribkoff *et al.*, 2001). We investigated this possibility using *hSlo* (α subunit) BK channels expressed in human embryonic kidney cells and patch-clamping. We discovered that compound A potently inhibits BK channel-activation at nanomolar concentrations in inside-out membrane patches. BK channel currents activated by depolarising voltage pulses in the presence of 10 μM free calcium were inhibited by compound A in a concentration-dependent manner. 100 nM compound A completely inhibited outward potassium currents in less than one minute. The concentration that produced half maximal inhibition was approximately 3 nM, indicating a high apparent affinity for BK channels. This is the first time a molecular site of action has been determined for a compound of this structural class and identifies a novel BK channel blocker.

Gribkoff V.K., Starrett J.E., Jr. & Dworetzky S.I. (2001) *Neuroscientist*, 7:166-77.

Estimations of relative anion-cation permeabilities deduced from reversal (dilution) potential measurements, as in glycine receptor channel studies, are essentially model independent

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In a recently completed series of structure-function studies on human recombinant glycine receptor (GlyR) channels, expressed in HEK293 cells, we have shown that a single, double (SDM) and triple (STM) point mutations in the M2 region of the glycine receptor were able to each switch the GlyR selectivity from being anion- to cation-selective (Keramidas *et al.*, 2000, 2002; Moorhouse *et al.*, 2002). In order to relate ion selectivity to changes in electrostatic effects in the channel pore and in its minimum pore diameter, we needed both anion-cation permeability ratios and minimum pore diameters. The latter were determined by measuring cation-cation (or anion-anion) permeability ratios for a series of large test cations (anions) for the different cation-selective (anion-selective) mutant GlyRs. They were determined from bionic potentials, measuring the change in reversal potential, under whole cell patch clamp conditions, when the external solution cation (anion) was substituted by various larger cations (anions) for cation selective (anion-selective) mutant GlyRs. It has been shown that for such bionic measurements, the form of the membrane potential equation is essentially independent of the mathematical model underlying it, as discussed in Barry & Gage (1984).

However, concern has been expressed about the validity of using the Goldman-Hodgkin-Katz (GHK) equation (see Barry & Gage, 1984) for dilution potential measurements, given the inherent assumptions (a constant electrical field in the membrane and independence of ion fluxes) in its derivation. Experimentally, the anion-cation permeability ratios (P_{Cl}/P_{Na}) were determined from dilution potentials by measuring the change in reversal potential, when the external solution NaCl concentration was decreased to about 50% and then to about 25% and each shift in reversal potentials plotted against external NaCl activity. Experimentally, it was noted that the data did fit the GHK equation with the predicted straight line and constant permeability ratio. The GHK equation is:

$$\Delta V_{rev} = RT/F \ln [a_{Na}^{o} + (P_{Cl}/P_{Na})a_{Cl}^{i}] / [a_{Na}^{i} + (P_{Cl}/P_{Na})a_{Cl}^{o}]$$

where ΔV_{rev} is the shift in reversal potential, R, T and F have their usual significance and a_{Na} and a_{Cl} represent the activities of Na^+ and Cl in the external (o) and internal (i) solutions respectively.

We then fitted the data to the Planck equation, derived by solving the Nernst-Planck flux equations, which has virtually opposite underlying assumptions (a non-constant electrical field and a macroscopic electroneutrality condition) to the GHK ones. The Planck equation is:

$$\Delta V_{rev} = (RT/F) \ (P_{Na} \ P_{Cl}) / (P_{Na} + P_{Cl}) \ \ln \ a_{NaCl}^{o} / a_{NaCl}^{i}$$

However, it produced very similar permeabilities to those of the GHK equation. For example, P_{Cl}/P_{Na} values using the GHK [Planck] equation for the SDM and STM cation-selective mutant GlyRs were 0.12 [0.14] and 0.27 [0.27] and P_{Cl}/P_{Na} for the anion-selective WT GlyR was 28.5 [26.2].

Hence, the anion-cation permeability ratios determined using the GHK or Planck equations are essentially independent of the limiting underlying assumptions of those equations.

Barry, P.H. & Gage, P.W. (1984) In: *Current Topics in Membranes and Transport, 21,* ed. Stein, W.E. pp. 1-51. Orlando: Academic Press.

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Keramidas, A., Moorhouse, A.J., Pierce, K.D., Schofield, P.R. & Barry P.H. (2002) *Journal of General Physiology*, 119, 393-410.

Moorhouse, A.J., Keramidas, A., Zaykin, A., Schofield, P.R. & Barry P.H. (2002) *Journal of General Physiology,* 119, 411-425.

Role of charged residues in coupling ligand binding and channel activation in the extracellular domain of the glycine receptor

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The glycine receptor is a member of the ligand gated ion channel receptor superfamily that mediates fast synaptic transmission in the brainstem and spinal cord. Following ligand binding, the receptor undergoes a conformational change that is conveyed to the transmembrane regions of the receptor resulting in the opening of the channel pore. Using the acetylcholine binding protein structure as a template, we modelled the extracellular domain of the glycine receptor α-1 subunit and identified the location of charged residues within loops 2 and 7 (the conserved Cys-loop). These loops have been postulated to interact with the M2-M3 linker region between the transmembrane domains 2 and 3 as part of the receptor activation mechanism. Charged residues were substituted with cysteine, resulting in a shift in the concentration-response curves to the right in each case. Covalent modification with 2-trimethylammonioethyl methanthiosulfonate was demonstrated only for K143C, which was more accessible in the open state than the closed state, and resulted in a shift in the EC50 towards wild-type values. Charge reversal mutations (E53K, D57K and D148K) also impaired channel activation, as inferred from increases in EC50 values and the conversion of taurine from an agonist to an antagonist in E53K and D57K. Thus, each of the residues E53, D57, K143 and D148 are implicated in channel gating. However, the double reverse charge mutations E53K:K276E, D57K:K276E and D148K:K276E did not restore glycine receptor function. These results indicate that loops 2 and 7 in the extracellular domain play an important role in the mechanism of activation of the glycine receptor.

APPS/PSNZ Meeting - Sydney 2003

APPS Lecture - David Allen

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CHAIR

Skeletal muscle function: the role of ionic changes in fatigue, damage and disease

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Repeated activity of skeletal muscle changes its properties in a variety of ways; muscles become weaker with intense use (fatigue), may feel sore and tender after excessive use, and can degenerate in many disease conditions. Early ionic changes are critical to the development of each of these conditions.

Central to this experimental approach has been the development of the single fibre preparation of mouse muscle. Individual cells can be dissected with intact tendons and stimulated to produce force. Fluorescent indicators can be micro-injected into the fibres and intracellular Ca^{2+}, Na^{+}, pH, Mg^{2+}, ATP etc can all be measured from one cell whilst simultaneously monitoring the mechanical performance. Other substances can be injected into the cells (proteins, peptides, caged compounds, plasmids etc) and after activity the cell can be prepared for immunohistochemistry, light microscopy, electron microscopy etc.

In 1988 when we started this work, the dominant theory was that intracellular acidosis caused muscle fatigue. In contrast we found that single fibres could fatigue with little or no pH change (Westerblad & Allen, 1992) but failure of calcium release was found to be a major cause of fatigue (Westerblad & Allen, 1991). Currently we propose that precipitation of calcium and phosphate in the sarcoplasmic reticulum contributes to the failure of calcium release (Allen & Westerblad, 2001).

Muscles can be used to shorten and produce force or they can be used to deaccelerate loads (eccentric contractions). A day after intense eccentric exercise muscles are weak, sore and tender and this damage can take a week to recover. In this condition sarcomeres are disorganised and there are increases in resting Ca^{2+} and Na^{+} (Balnave & Allen, 1995; Yeung *et al.*, 2003). Recently we discovered that the elevation of Na^{+} occurs through a stretch-activated channel which can be blocked by either gadolinium or streptomycin. Preventing the rise of $[Na^{+}]_i$ with gadolinium also prevents part of the muscle weakness after eccentric contractions (Yeung *et al.*, 2003).

Duchenne muscular dystrophy is a lethal degenerative disease of muscles in which the protein dystrophin is absent. Dystrophic muscles are more susceptible to stretch-induced muscle damage and the stretch-activated channel seems to be one pathway for the increases in intracellular Ca^{2+} and Na^{+} which are a feature of this disease. We have recently shown that blockers of the stretch-activated channel can minimize some of the short-term damage in muscles from the *mdx* mouse, which also lacks dystrophin (Yeung *et al*., submitted). Currently we are testing whether blockers of the stretch-activated channels given systemically to *mdx* mouse can protect against some features of this disease.

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