APPS/PSNZ Meeting - Sydney 2003

Free Communications 6: Ion channels and membrane transport

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Chair: David Saint

Involvement of a voltage-dependent calcium channel in signal transduction in the 2-cell embryo

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Platelet-activating factor (PAF) is an autocrine trophic factor for the preimplantation embryo that induces a transient increase in $[Ca^{2+}]_i$ in the 2-cell embryo. The $[Ca^{2+}]_i$ transient had an absolute requirement for influx of external calcium and was inhibited by blockers of L-type calcium channel blockers but not by a variety of non-L-type channel blockers. This study used whole cell patch clamp methodology to assess whether the early mouse embryo expressed a functional calcium channel with the properties of an L-type channel.

Pre-implantation mouse embryos were recovered after superovulation of female QS mice by intraperitoneal injections of equine chorionic gonadotrophin (10 i.u.) followed 48 hours later by human chorionic gonadotrophin (10 i.u.) and mating. Mice were killed by cervical dislocation and 2-cell embryos were flushed from the reproductive tract into Hepes-modified HTF medium containing 3 mg/ml BSA. The zona pellucida was removed by brief treatment with 0.5% pronase. Standard whole-cell patch-clamp techniques were used to study Ca^{2+} currents in two-cell embryos. The membrane potential was held at -60mV and depolarising voltage pulses of 1s duration were applied between -20 and +80 mV at intervals of 5 s. Currents were low-pass filtered, sampled and digitised at 0.2 kHz. Ba^{2+} was used as the charge carrier. The currents at each voltage-step were recorded before and after treatment of embryos with different kinds of L-type Ca^{2+} channel blockers: diltiazem (75 μM), nifedipine (80 μM) and verapamil (80 μM). Inward currents were measured as the difference between the whole cell currents before and after the addition of a drug or control to the bath solution, consisting of NaCl 55mM, KCl 4.69mM, $MgCl_2$ 0.2mM, Na_2EDTA 0.11mM, glucose 5mM, $CaCl_2$ 2.04mM (1.94 mM free Ca2+), Hepes 20.4mM, $BaCl_2$ 50mM (49.99 mM free Ba^{2+}), adjusted to pH 7.4, 300 mosM/kg.

Using diltiazem, a current of 0.23 ± 0.03 nA (mean ± SEM) was detected and was maximal at a voltage of 36.94 ± 2.59 mV. A similar current was evident when either nifedipine or verapamil were used. Prior treatment of embryos with exogenous PAF resulted in a significant ($P < 0.05$) reduction in the proportion of embryos expressing the current and the size of the current compared with those pre-treated with rPAF acetylhydrolase. The results show that 2-cell embryos possess a depolarisation-activated membrane channel, with the properties of an L-type calcium channel. The desensitisation of channel activity by prior PAF challenge suggests that the current was activated during PAF-induced calcium signalling.

Negative feedback inhibition of Ca^{2+} influx during P_{2Y2} receptor activation

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G protein-coupled receptors transduce extracellular stimuli via G proteins to intracellular effectors resulting in the activation of second messenger signalling cascades mediated by Ca^{2+} or cAMP. In HT29 human colonic epithelial carcinoma cells, the activation of the M_3 muscarinic receptor by carbachol (CCh) results in a Ca^{2+} response with a characteristic prolonged plateau phase that occurs due to Ca^{2+} influx following activation of store-release sensitive channels in the plasma membrane. In contrast, the Ca^{2+} response following the activation of the P_{2Y2} purinergi receptor shows no plateau phase (Cummins *et al*., 2000). Both of these responses are pertussis toxin insensitive and mediated by G proteins of the G_q family. These data suggest that during P_{2Y2} activation, the lack of a plateau phase may result from the inhibition of Ca^{2+} influx. The small G proteins Rac and Cdc42 are known to be involved in Ca^{2+} signalling pathways (Peppelenbosch *et al*., 1996; Djouder *et al*., 2000) and protein kinase C (PKC), a known target of Cdc42 (Slater *et al*., 2001), has also been reported to regulate the Ca^{2+} signalling pathway (Petersen & Berridge, 1994; Lee *et al*., 1997). The aim of this present study was to investigate the roles of Rac, Cdc42 and PKC in the inhibition of Ca^{2+} influx during P_{2Y2} receptor activation.

Standard Fura-2 imaging techniques were used to monitor changes in intracellular Ca^{2+} concentration in HT29 cells. Ca^{2+} influx was monitored using the rate of quenching of the Fura-2 signal by exogenous Mn^{2+}. Replication-deficient adenoviruses expressing the cDNA encoding either wild type (wt), dominant negative (dn) or constitutively active (ca) mutants of Rac, Cdc42 and PKCα were created using standard techniques (Cummins *et al*., 2000).

In HT29 cells exposed to UTP, the rate of Mn^{2+} influx as measured by Fura-2 quenching was 66% of the influx rate in response to CCh, indicating that the lack of a plateau phase during UTP exposure was due to reduced influx. When HT29 cells were infected with adenoviruses expressing dnRac or dnCdc42, the rates of Mn^{2+} influx increased to those observed with CCh stimulation. Furthermore, when the cells were infected with caRac, Mn^{2+} influx induced by CCh was reduced to below control levels. These indicate the involvement of both Rac and Cdc42 in the negative feedback inhibition of UTP mediated Ca^{2+} influx. Co-infection of dnCdc42 and caRac returned Mn^{2+} influx to the levels observed in control cells with UTP stimulation, indicating that Rac is upstream of Cdc42.

In HT29 cells infected with an adenovirus expressing wtPKCα, there was no change in the rates of Mn^{2+} influx in the presence of UTP. In contrast, when the cells were infected with a dnPKCα adenovirus, the rate of Mn^{2+} influx was increased to the levels observed with CCh, indicating a role for PKCα in the control of Ca^{2+} influx in these cells. To determine whether PKCα was acting via the same signalling pathway as Rac/Cdc42, HT29 cells were treated with Toxin B, an inhibitor of Rac/Cdc42, and dioleylglycerol, an activator of PKC. This resulted in an inhibition of Mn^{2+} influx to levels similar to that observed with UTP, indicating that the effect of blocking Rac/Cdc42 could be overcome by activating PKC, demonstrating that PKCα is downstream of Rac/Cdc42. This study shows that the main difference in the muscarinic and purinergic Ca^{2+} responses in HT29 cells, is due to a Ca^{2+} influx negative feedback pathway that is activated by P_{2Y2} receptors.

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Calcium release-activated calcium current in rat hepatocytes

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Store-operated Ca^{2+} (SOC) channels play a central role in regulating intracellular Ca^{2+} concentration in hepatocytes and other nonexcitable animal cells (Gregory & Barritt, 2003). A major function of SOC channels appears to be to replenish intracellular Ca^{2+} stores when intracellular Ca^{2+} is lost from the cell during agonist-induced increases in the cytoplasmic Ca^{2+} concentration. One of the best-known store operated channels, Ca^{2+}release-activated Ca^{2+} (CRAC) channel has been extensively characterised in a number of immortalised cell lines. There is little evidence, however, that I_{CRAC} is activated in the physiological conditions in cells in primary culture. It has been speculated that the highly Ca^{2+}-specifi c CRAC channels are only expressed in blood cells and in transformed cells.

The aim of the present experiments was to elucidate the properties of the SOC channels in rat hepatocytes. Hepatocytes were isolated by collagenase digestion and plated on glass coverslips. Patch-clamp recording was conducted in the whole-cell mode using standard procedures after 24-48 hours.

Depletion of intracellular Ca^{2+} stores in rat hepatocytes activated a Ca^{2+}-selective inward current. Properties of this current, including high selectivity for Ca^{2+}, strong inward rectifi cation, fast Ca^{2+} dependent inactivation at negative potentials and block by La^{3+} and 2-APB, were similar or identical to those of I_{CRAC} found in mast cells, RBL cells, Jurkat T lymphocytes (Zweifach & Lewis, 1993; Hoth & Penner, 1992; Bakowski & Parekh, 2002) and H4-IIE liver cells (Rychkov *et al.* 2001). The amplitude of I_{CRAC} in rat hepatocytes varied between −30 and −120 pA at −100 mV with an average density of about −1 pA/pF. Extracellular application of vasopressin or ATP activated a current with the same properties and the same size as that observed by $InsP_3$ induced depletion of the stores. I_{CRAC} developed more slowly with vasopressin than with ATP as agonist. Increasing the concentration of ATP shortened the delay in the development of I_{CRAC}, but did not change the amplitude of the current or the rate of its development. Concentrations of ATP (5-10 µM) that cause waves of increased cytoplasmic Ca^{2+} concentration also activated I_{CRAC}. So far, no other type of current activated by Ca^{2+} store depletion has been detected in these cells. It is concluded that CRAC channels are the major, and possibly the only, type of SOC channel in rat hepatocytes.

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Characterisation of chloride currents in the mouse pre-implantation embryo

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Pre-implantation embryonic development describes the process by which the embryo grows from the zygote (one-cell) to the blastocyst (64-254 cells) after which it implants into the placenta. Blastocysts are composed of two different cell types, the inner cell mass (ICM) and trophectoderm cells (TE). Chloride currents throughout these stages of embryonic development are not well characterised. A swelling activated Cl^- current was shown to be cell cycle dependent as well as developmentally regulated however this current was not examined in isotonic solutions (Kolajova *et al.*, 2001). In the blastocyst it is believed that Cl^- is transported by both paracellular (Manejwala *et al.*, 1998) and transcellular mechanisms (Brison & Leese, 1993) and that the expansion of the blastocoel cavity is largely reliant on Cl^- channels and a Cl^-/HCO_3^- exchanger (Zhao *et al.*, 1997). The cystic fi brosis transmembrane conductance regulator (CFTR) was recently shown to be present in human blastocysts and it may play a role in the process described above (Ben-Chetrit *et al.*, 2002). This study aimed to characterise Cl^- currents observed in isotonic conditions in the pre-implantation embryo by looking at the eight distinct members of the voltage gated chloride channel family (ClC) (1-7 and K) as well as CFTR.

The mRNA expression pattern of CFTR and ClC channels in the early mouse embryo was determined by RT-PCR. The channels observed in the pre-implantation embryo were ClC-2 to ClC-7, ClC-K and CFTR. Furthermore, ICM and TE cells were separated and RT-PCR of CFTR was carried out for each cell type. The results showed that CFTR mRNA is only present in TE cells. These data suggest that Cl^- channels may play an important roles in the pre-implantation embryo.

The whole-cell patch-clamp technique was used in order to characterise Cl^- currents in the mouse pre-implantation embryo. In the late four-cell stage two major currents were identifi ed through the use of various Cl^- channel antagonists. These included a DIDS-sensitive (non-specifi c Cl^- channel blocker) and glibenclamide-sensitive (CFTR blocker) current. DIDS inhibited approximately 46% and glibenclamide 38% of the Cl^- current. When both drugs were added simultaneously, the Cl^- current was reduced by approximately 74% indicating that the DIDS and glibenclamide sensitive currents are individual currents. In the ICM glibenclamide had no effect on the Cl^- current whereas in a 3.5 day trophoblast cell line preliminary results indicate that there is a large glibenclamide sensitive current. These electrophysiological results are consistent with the CFTR mRNA expression pattern observed.

The exact role that Cl^- channels play in the pre-implantation embryo still remains to be identifi ed. The results described above show that CFTR along with other ClC channels are present in the pre-implantation embryo at the mRNA level and that they are most likely to be responsible for the currents observed.

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Clustering of recombinant $GABA_A$ receptors alters channel properties

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'Native' $GABA_A$ receptors display distinct electrophysiological properties not always seen in recombinant receptors irrespective of subunit composition. Native channels can have conductances over 40pS (Gray & Johnson, 1985; Smith *et al.*, 1989; Curmi *et al.*, 1993). Moreover, the conductance of some channels can be increased by modulating drugs such as diazepam, pentobarbitone and propofol (Eghbali *et al.*, 1997; Guyon *et al.*, 1999; Eghbali *et al.*, 2003). By contrast, conductances of recombinant channels have never exceeded about 30pS and, although their open probability can be increased by modulating drugs, conductance is not enhanced by drugs.

It has been suggested that high channel conductances may represent cooperative openings of clustered channels resulting in an apparent high single channel conductance. We tested this hypothesis in an expression system by co-expressing two proteins known to cluster $GABA_A$ receptors. Rapsyn is a membrane associated protein that plays a crucial role in clustering ACh receptors at the neuromuscular junction, but has also been shown to cluster expressed $GABA_A$ receptors. GABARAP interacts with the $GABA_A$ γ subunit and promotes receptor clustering (Wang *et al.*, 1999).

We co-transfected (lipofectin) $GABA_A$ α5 and β1 subunit cDNAs with or without rapsyn into mouse fibroblast L929 cells. We measured single channel conductance in the cell-attached (c/a) or inside-out (i/o) configurations 24-72 hours later. In the control groups (i.e. $GABA_A$ subunits alone), single channel conductances were within the range 10-35pS. When rapsyn was co-expressed with $GABA_A$ subunits, 4 out of 8 patches showed single channel conductances greater than 40pS. Control patches expressing $GABA_A$ α1, β1 and γ2s subunits alone had a mean conductance of 22.3 ± 1.2pS (n=15). In 16 out of 25 patches recorded from cells co-transfected with $GABA_A$ α1,β1 and γ2s subunits and GABARAP, single channel conductances were above 40pS (γ=60.7 ± 4.3pS, n=16). These 'high' conductance channels were never seen in control patches. High and low conductance channel activity was blocked by 100μM bicuculline. The current-voltage relationship of high conducting channels showed outward rectification of the current, similar to that seen in native receptors.

Diazepam can increase both open probability and conductance of $GABA_A$ channels containing the γ subunit. In 5 patches from cells co-transfected with $GABA_A$ α1,β1 and γ2s subunits and GABARAP, both of these effects were seen irrespective of initial channel conductance. In control patches where GABARAP was not expressed, diazepam did not increase channel conductance.

Immunofluorescent studies revealed that coexpression of rapsyn or GABARAP with $GABA_A$ subunits, showed a punctate pattern of staining of surface receptors compared to a diffuse pattern in control cells.

Our results show that co-expression with "clustering" proteins can change the properties of recombinant $GABA_A$ channels. It is possible that clustered receptors may be able to couple and open cooperatively by virtue of their close physical proximity.

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APPS/PSNZ Meeting - Sydney 2003

Invited Lecture - Julian Paton

Tuesday 30th September 2003

Chair: Peter Gage

Signalling across the blood brain barrier: Implications for blood pressure control

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Our long-term goal is to understand cellular signalling mechanisms involved in the etiology of essential hypertension. Our hypothesis is that this disease may arise, in part, from changes within brainstem circuits controlling arterial pressure, and in particular to occlusion of arterial baroreceptor afferent information at the level of the primary afferent relay within the brainstem. Although it is established that baroreceptors regulate arterial pressure on a moment-to-moment basis, they may also control it long term (Thrasher, 2002). It follows then that desensitisation of this reflex circuit could contribute to high levels of blood pressure. I will discuss the central actions of angiotensin II on neuronal circuitry dedicated to controlling the baroreceptor reflex. Based on *in vivo* somatic gene transfer studies to identify intracellular signalling pathways, and dynamic confocal calcium imaging from cells within the nucleus of the solitary tract (NTS), we hypothesise a novel form of inter-cellular communication, one of *vascular-neuronal signalling*. Our model includes a process whereby angiotensin II stimulates nitric oxide release from the endothelium, which crosses the blood brain barrier to modulate adjacent inhibitory synaptic processes and shunts out incoming afferent information from arterial baroreceptors. Such a signalling process is consistent with that described for the control of GnRH within the median eminence (Prevot *et al.*, 2000). Moreover, using focal genetic approaches to chronically block endothelial cell derived nitric oxide results in an augmentation of baroreceptor reflex function and a fall in arterial pressure towards control levels in a rat model of hypertension. I will demonstrate that the specificity of action of nitric oxide on inhibitory (GABA) transmission in the NTS likely relates to the low concentration of the gas and/or proximity of the nitric oxide synthase isoform to its target (Paton *et al.*, 2002). In conclusion, activation of endothelial nitric oxide synthase within the NTS, which can be induced by physiological levels of angiotensin II, plays a major role in regulating cardiovascular function. Hyperactivity of angiotensin II and/or endothelial nitric oxide synthase within this nucleus may contribute to the persistent elevation of arterial pressure as observed in essential hypertension.

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