C75

Voltage-dependent inhibition of recombinant NMDA receptor-mediated currents by 5-hydroxytryptamine

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Glutamate activated NMDA receptors mediate excitatory synaptic transmission in the central nervous system and play an important role in many physiological processes including long term potentiation, synaptogenesis and excitoxicity. The modulation of NMDA-mediated responses by 5-hydroxytryptamine (5-HT) has been reported in spinal cord motorneurones (Chesnoy-Marchais & Barthe, 1996; MacLean & Schmidt, 2001) and this interaction has been implicated in the control generation of motor rhythm activity in the mammalian spinal cord. In the present study, the effect of 5-HT on heteromeric recombinant NMDA receptors (NR1a+2A, NR1a+2B and NR1a+2C) expressed in *Xenopus* oocytes was investigated using the two-electrode voltage clamp recording technique. Stage V-VI oocytes were removed from anaesthetized female *Xenopus laevis* in accordance with the guidelines of the University of Queensland Animal Experimentation Ethics Committee. In the absence of external Mg^{2+} ions, 5-HT inhibited NMDA receptor-mediated currents evoked with 100 μM glutamate and 10 μM glycine in a concentration-dependent manner. The inhibitory effect of 5-HT was strongly voltage-dependent whereby 40 mV depolarization shifted the concentration-response curve to the right and increased the half-maximal inhibitory concentration (IC_{50}) approximately ten-fold. The IC_{50}'s obtained for 5-HT block of NR1a+2A, NR1a+2B and NR1a+2C subunit combinations expressed in oocytes voltage clamped at −120 mV were 60, 50 and 40 μM, respectively, and increased to 500 μM for NR1a+2A and 300 μM for NR1a+2B and NR1a+2C in oocytes held at −70 mV. Current-voltage curves obtained using voltage ramps applied during steady-state glutamate responses markedly rectified in the presence of 5-HT whereby inhibition of glutamate-evoked currents by 5-HT increased with membrane hyperpolarization. The voltage sensitivity of the inhibition (e-fold change per 20 mV change in membrane potential) indicates a block by 5-HT deep within the membrane electric field. The 5-HT precursor, tryptamine inhibited glutamate-evoked currents through NMDA receptors with a similar potency whereas tryptophan (≦1 mM) failed to inhibit the response. Taken together, these data suggest that 5-HT and related compounds can attenuate glutamate-mediated excitatory synaptic responses and may provide a basis for drug treatment of excitotoxic neurodegeneration.

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This work was supported by the Australian Research Council.

C76

SLURP-1, a secreted mammalian protein modulates α7 human nicotinic acetylcholine receptors.

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The Ly-6/uPAR proteins are a subfamily of urokinase-type plasminogen activator receptors (uPAR) characterized by a distinct disulfide bonding pattern between 8 or 10 cysteine residues and by a conserved pattern CCXXXXCN. The Ly-6/uPAR family can be further subdivided based on the presence or absence of a GPI-anchoring signal sequence. Ly-6/uPAR proteins attached to the cell membrane by a carboxy-terminal linker include Lynx-1 whereas secreted proteins include secreted mammalian Ly-6/uPAR-related protein 1 (SLURP-1) and SLURP-2, which is an isoform of Lynx-1. The amino acid sequence of SLURP-1 has some homology with the secreted snake α-neurotoxins, suggesting that its 3-dimentional structure may also be similar. Mutations in the gene coding for SLURP-1 in humans have been reported in individuals suffering from Mal de Meleda, a rare autosomal recessive skin disorder, characterised by hyperkeratosis on the palms of the hands and soles of the feet. Snake α-neurotoxins are known to interact with the muscle and neuronal subtypes of the nicotinic acetylcholine receptor (nAChR). α7 nAChRs have been reported to be involved in homeostasis and differentiation of epidermal keratinocytes. We assessed the effect of SLURP-1 at human α7 nAChRs expressed in *Xenopus* oocytes. Preincubation with SLURP-1 increased the amplitude of the ACh-evoked currents in a concentration dependent manner suggesting that SLURP-1 may exert its effects *in vivo, via* nAChRs where it acts as a signal in normal growth and development of keratinocytes. Mutations in the gene coding for SLURP-1, such as frame shift or introduction of a stop codon are expected to disrupt this action.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C77

2-Aminoethoxydiphenyl borate blocks human TRPC5 channels via an extracellular site

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Mammalian counterparts of the Drosophila trp genes are suggested to encode Ca^{2+} permeable cationic channels such as store- and receptor-operated channels (Clapham et al. 2003). TRPC5 is one of the members in the canonical TRP (TRPC) family, which is widely expressed in the cardiovascular (Xu & Beech, 2001), nervous (Greka et al. 2003) and gastrointestinal systems (Lee et al. 2003). Human TRPC5 was cloned in 1999 (Sossey-Alaoui et al. 1999) but its function and pharmacological profile are lacking. 2-aminoethoxydiphenyl borate (2-APB) is a widely used agent in

the studies of IP3 receptor and store-operated channel signaling. In this study we used patch clamp recordings and Ca^{2+} imaging to examine the effects of 2-APB on human TRPC5 cationic channel stably expressed in HEK 293 cells under a tetracycline-regulated promoter. The experiments were performed at room temperature. Data are expressed as mean ± S.E.M. The unpaired student's *t* test was used. The ionic current mediated by TRPC5 was activated by Gd^{3+} at 10 μM. 2-APB inhibited the whole-cell TRPC5 current in a reversible and concentration-dependent manner (IC_{50} = 19 μM, n = 6). Bath-applied 2-APB (75 μM) also blocked TRPC5 in outside-out (current value at −80 mV was decreased by 67.3 ± 6.9 %, n = 3, P = 0.01) but not inside-out membrane patches (6.2 ± 9.8 %, n = 8, P > 0.05), suggesting an external binding site. Intracellular dialysis of cells with 150 μM 2-APB did not block TRPC5 and extracellular 2-APB remained effective (n = 3). In fura-PE3 Ca^{2+} imaging experiments, methoxyverapamil (D600) at 10 μM (n = 60 cells, P > 0.05) or nifedipine at 1 μM (n = 99 cells, P > 0.05) had no effects on TRPC5.The data indicate 2-APB inhibits human TRPC5 channel via the extracellular surface and Ca^{2+} antagonists have no effects on the channel. The presence of extracellular 2-APB binding site, which is distinct from the Ca^{2+} antagonist binding sites of voltage-gated Ca^{2+} channel, may be useful as a new drug target of TRPC5.

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This work was funded by The Wellcome Trust. We are grateful to A.K. Srivastava for human TRPC5 DNA fragments.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C78

Antibody to the predicted outer pore of TRPC5 ablates calcium entry evoked by store-depletion in isolated rabbit arterioles

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Almost a quarter of a century ago Casteels & Droogmans (1981) published their seminal work on arterial smooth muscle showing that depletion of intracellular stores by noradrenaline stimulated the rate of calcium uptake. The pathway was resistant to conventional calcium antagonists and inhibited by potassium-induced depolarisation. Subsequently many studies have shown this is a common phenomenon in smooth muscle but the underlying mechanisms and functional importance remain uncertain. Evidence has however accumulated that TRPC1 protein, a mammalian homologue of Drosophila transient receptor potential, is a component of the channel (Beech et al 2003). We have studied the phenomenon in rabbit pial arterioles and support a role for TRPC1 (Xu & Beech 2001). Rabbits were killed humanely according to approved schedule 1 procedures. Intriguingly, however, TRPC1 forms heteromultimeric channel assemblies with TRPC5 (Strubing et al 2001), which is potentially important because there is TRPC5-encoding mRNA in pial arterioles (Flemming et al 2003). Therefore, we tested the hypothesis that TRPC1 operates in concert with TRPC5 in the native store-operated calcium-entry mechanism of arterioles. In order to test for TRPC5 protein, three new anti-TRPC5 antibodies were prepared. All antibodies labelled over-expressed TRPC5 and specifically bound a protein of the same mass in blood vessels. There was evidence of glycosylated TRPC5 and plasma membrane localisation. One of the antibodies was designed to a unique amino acid sequence in the predicted outer vestibule of the ion pore of TRPC5. Strikingly, the antibody ablated calcium-entry through TRPC5 over-expressed in HEK-293 cells and calcium-entry evoked by store depletion in arterioles, without effect on background calcium entry. The antibody also suppressed a stimulatory effect of lanthanum in store-depleted arterioles, consistent with the unusual response of TRPC5 to this lanthanide. We demonstrate the antibody is highly specific for TRPC5, being able to distinguish it from the closely related proteins TRPC1, TRPC4 and TRPC6. From these data it is suggested that TRPC5 protein is expressed in vascular smooth muscle and that we have engineered a novel tool for the study of its function. Most significantly we demonstrate TRPC5 is an additional and critical subunit of the physiological store-operated calcium-entry mechanism in vascular smooth muscle.

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Supported by the Wellcome Trust.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C79

Flufenamic acid is a pH-dependent antagonist of TRPM2 channels

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TRPM2, a member of the melastin related TRP (transient receptor potential) channel family, is widely and most abundantely expressed in the brain (Kraft et al. 2003) but is also found in pancreatic beta cell-lines (Herson et al. 1999). TRPM2 forms a Ca^{2+}-permeable, non-selective, cation channel activated by species generated by oxidant stress, including intracellular ADP-ribose, hydrogen peroxide, and β-NAD^+. To date no potent antagonists of this channel have been described.HEK293 cells expressing tetracycline-inducible Flag-tagged TRPM2 (TRPM2-HEK293 cells) and CRI-G1 rat insulinoma cells were grown under standard tissue culture conditions. TRPM2 expression was induced by incubating cells for 24h with 1μg/ml tetracycline. All experiments were performed at room temperature using the whole cell variant of the patch clamp technique. Data are presented as mean ± S.E.M.When 100μM ADP-ribose was included in the patch pipette solution TRPM2-HEK293 exhibited a large inward current at −70 mV. This current was not present in parental HEK-293 cells and,

characteristically of TRPM2, could be eliminated by removal of extracelluar Ca^{2+}. Extracellular application of FFA (50-1000 μM) produced complete block of this TRPM2-mediated current. The rate of development of this block was dependent on FFA concentration (time to half-maximal block ($t_{1/2}$) 91.6±1.6s (50μM, n=3), 40.4±4.1s (100μM, n=7), 13.1±2.3s (200μM, n=4), 2.6±0.4s (500μM, n=3), and 1.5±0.5s (1mM, n=3), and appeared voltage-independent. Unlike the current inhibition produced by removal of extracellular Ca^{2+}, the majority of FFA-mediated block was irreversible. The kinetics of antagonism by 100μM FFA were pH-dependent (e.g. pH 6 $t_{1/2}$=3.8±0.4s; pH 7.4 $t_{1/2}$=40.4±4.1s, n=7; pH8 $t_{1/2}$=135.9±27.4s, n=3). Furthermore antagonism seemed to require the channel to be activated. In corresponding experiments performed on CRI-G1 cells, FFA (50-1000 μM) produced a similar antagonism of ADP-ribose induced currents. The time to half maximal block was somewhat faster than for HEK293-TRPM2 cells ($t_{1/2}$=21.8±1.0s (50μM, n=4), 14.6±3.8s (100μM, n=5), 5.3±1.9s (200μM, n=4), 3.9±0.6s (500μM, n=4), 1.5±0.2s (1 mM, n=3), and could be speeded further by lowering the external pH to 6.0 ($t_{1/2}$=3.8±0.6s (n=4), 100 μM FFA). Unlike TRPM2-HEK293 cells the majority of the block observed in CRI-G1 cells was readily reversed on FFA removal.

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K.H. is in receipt of of EU Framework V Postdoctoral Fellowship, HEK293-TRPM2 cell line is a kind gift of A.Scharenberg

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C80

Human TRPC5 channel activated by a multiplicity of signals in single HEK-293 cells

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Human TRPC5 (hTRPC5) is a putative cationic channel that was cloned from a region of X chromosome associated with mental retardation (Sossey-Alaoui *et al*, 1999). We produced hTRPC5 stably expressing HEK-293 cells under tetracycline-regulation. Over-expression of hTRPC5 was confirmed by real time RT-PCR and western blotting. Functional hTRPC5 were measured by calcium imaging and electrophysiology. In tetracycline-induced cells, no basal activity was evident but activity was induced by carbachol stimulation of muscarinic receptors independently of calcium release. This is "receptor-activation", as described for mouse TRPC5 (Strubing *et al*, 2001). In addition, and in the absence of receptor stimulation, extracellular 0.1 mM gadolinium activated TRPC5, an effect that was mimicked by 5-10 mM extracellular calcium with intracellular calcium buffered. We refer to this as "external ionic-activation". Using whole-cell recording, we demonstrated that TRPC5 was also activated by modest elevation of $[Ca^{2+}]_i$ in the absence of GTP - "calcium-activation". A putative fourth activation mechanism is a signal from depleted intracellular calcium stores. Consistent with this idea, hTRPC5 was activated by a standard protocol in which calcium stores were first depleted by treatment of cells with thapsigargin in calcium free solution, followed by re-addition of 1.5 mM calcium to the bath solution. This result can be mimicked by replacing calcium with 0.5 mM barium, an effect that was difficult to explain by calcium activation. TRPC1 is thought to participate in a physiological heteromultimeric assembly with TRPC5 (Hofmann *et al*, 2002). When it was co-expressed with hTRPC5, the store-operated property of hTRPC5 was retained, although the amplitude of the signal was smaller. We also tested different activation signals on isolated cells, and thus on the single expression levels of these cells. Multiplicity of TRPC5 activation was evident: the TRPC5-dependent "store-operated" calcium re-entry signal and "receptor-activation" both occurred in a single cell, as did "external-ionic" and "receptor" activation. In conclusion, hTRPC5 is activated by a range of stimuli, avoiding dependence on a single critical activator like TRPV4 (Vriens *et al*, 2004) and unlike many other ion channels.

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The work was supported by the Wellcome Trust, MRC, BBSRC, and Nuffield Hospital Leeds.

C81

Role of neuronal calcium sensor 1 protein (NCS-1) in human TRPC5 calcium channel activity

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The Ca^{2+}-dependent non-selective cation channel TRPC5 is a membrane protein with a prominent role in providing a route for Ca^{2+} entry into excitable cells. TRPC5 has been reported to regulate neurite extension and growth-cone morphology in hippocampal neurons (Greka *et al*, 2003). While the nature of the moieties initiating channel gating remain elusive, it is apparent that TRPC5 gating is complex and subject to regulation by many signals arising close to the plasma membrane (Strübing *et al*, 2001). Here we examine whether NCS-1 (Burgoyne & Weiss, 2001) plays a role in TRPC5 function in HEK293 cells, stably expressing tetracycline-regulated transcription of human TRPC5 (hTRPC5). $[Ca^{2+}]$i was monitored using Fura-PE3. Ca^{2+} signals from other cation channels were inhibited by 10 μM Gd^{3+}. The responses of hTRPC5 to a variety of activating signals were assessed in cells expressing either dominant-negative NCS-1 (E120Q; NCS1-DN) or dominant-negative calmodulin (no intact EF-hands; CaM DN). Data are presented as mean ± S.E.M. with *n* indicating the number of cells followed by the number of experiments in parentheses. Statistical significance was determined using an unpaired 2-tailed Student's *t* test. After depletion of intracellular Ca^{2+} stores with 1 μM thapsigargin, store- and receptor- operated hTRPC5 activity was assessed. Muscarinic receptors were activated by 100 μM carbachol. Gd^{3+} (100 μM) was used in separate experiments to activate hTRPC5 directly. All modes of hTRPC5 activity were significantly inhibited by NCS-1 DN ($p<0.001$; $n = 123$ (6-11)). By contrast, CaM DN expression resulted in no inhibition of hTRPC5 activity ($n = 49$ (3)). Mouse TRPC5 is known to be regulated by phospholipase C activity and we suggest a similar link for hTRPC5 because an inhibitor of phospholipase C, U73122 (10 μM), strongly inhibited the activity of hTRPC5. It seems plausible

that NCS-1 DN affected hTRPC5 because it displaced endogenous NCS-1 from one of its established protein partners, PI4 kinase (Zhao *et al*, 2001) thereby reducing PIP2 levels. We tested this idea by inhibiting PI4 kinase with wortmannin (10 μM; 30 min). Unexpectedly, we found that store-operated TRPC5 activity was unaffected after wortmannin treatment (n = 303 (2)). Therefore it seems unlikely that NCS-1 DN affects TRPC5 via PI4 kinase. We suggest NCS-1 plays a role in the function of hTRPC5, possibly mediating the stimulatory effect of Ca^{2+} on its channel activity.

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We acknowledge the support of the Wellcome Trust and thank Kate Beckingham (Rice University) for the CaM DN clone.

C82

Calmodulin regulation of calcium channels in bovine adrenal chromaffin cells.

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We have shown previously that N and Q type channels contribute to the voltage-operated Ca^{2+} current (VOCC) recorded in bovine adrenal chromaffin cells (from the abattoir) and that under prolonged stimulus conditions the exocytotic efficiency of the N-type channel is relatively reduced (Wykes et al 2001). We show that inactivation of the VOCCs in these cells is regulated by Ca^{2+}/Calmodulin, with the pharmacologically isolated N type channel displaying the most sensitivity.Inactivation was investigated using a train of depolarisations (50 x 10ms, from -80mV to +20mV at 20 Hz). Fractional inactivation was determined by normalising the peak amplitude of a current to that of the 1st current in the train. Results presented are the mean inactivation and s.e.m at pulse 50. Perforated patch recordings with extracellular Ca^{2+} produced inactivation of 0.48 +/- 0.02 (n=7), equimolar replacement with Ba^{2+} resulted in less inactivation, 0.86 +/0.03 (n=3). In whole cell experiments increasing the concentration of BAPTA in the electrode solution from 0.3mM to 10mM changed inactivation from 0.42 +/- 0.03 (n=7) to 0.74 +/- 0.03 (n=5). Channels were pharmacologically isolated to assess whether both subtypes possessed the same degree of inactivation. Inactivation of ω-CgTX GVIA (1μM) treated cells was 0.66+/- 0.05 (n=4), whereas for ω-Aga IVA (300nM) treated cells it was 0.32 +/- 0.02 (n=4). The difference in the pharmacologically isolated channel types was significant in an un-paired students t-test, p = 0.0006. Switching from Ca^{2+} to equimolar Ba^{2+} reduced the amount of inactivation observed for the N-type channel from 0.32 +/- 0.02 (n=4) to 0.89 +/- 0.04 (n=2).The molecular mechanisms underlying this effect were investigated. Inhibiting calcinuerin by 20 mins preincubation with 1μM cyclosporine A or by introducing 30μM calmodulin (CaM) inhibitory peptides through the patch pipette did not significantly reduce the level of inactivation. In contrast, adenoviral mediated expression of a mutant CaM deficient in Ca^{2+} binding (Alseikhan, B. A et al 2002) significantly reduced inactivation. In WT CaM expressing cells the inactivation was 0.39 +/- 0.02 (n=13), whereas inactivation in mutant CaM expressing cells was 0.64 +/- 0.03 (n=16) ($P<0.05$).Results are consistent with CaM acting directly to control N-type channel inactivation in adrenal chromaffin cells and could account for the reduced exocytotic efficiency observed with these channels during intense stimulation.

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This work was supported by the BBSRC, UK

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C83

Evidence that Kv3 voltage gated potassium channels influence neurotransmitter release from motor nerve terminals

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Voltage gated potassium (Kv) channels are critical to regulation of neurotransmitter release throughout the nervous system, but the roles and identity of the subtypes involved remain unclear. Here we show that Kv3 ion channels participate in regulation of transmitter release at the neuromuscular junction (nmj). Adult C57BL mice were killed humanely by CO2 exposure and the flexor digitorum brevis (FDB), transversus abdominus and lumbrical muscles dissected out and either aldehyde-fixed for light and electron microscopy or used for electrophysiological recordings. Light microscopic immunohistochemistry using antibodies raised in rabbit to specific sequences of Kv3.1-Kv3.4 subunits (Alomone Labs, 1:100-1:1000) revealed Kv3.3 and Kv3.4, but not Kv3.1b or Kv3.2, at neuromuscular junctions. The immunoreaction was co-localised with SV2 (Iowa hybridoma bank, 1;500) and was therefore likely to be within presynaptic terminals. Presynaptic localisation was confirmed by electron microscopy. To examine the roles of these Kv3 subunits intracellular recordings were made of evoked end plate potentials (EPPs) in FDB muscle fibres. Muscles were maintained in Ringers solution (composition in mM: NaCl, 120; NaH_2PO_4, 0.4; d-glucose, 5; $NaHCO_3$, 23.8; $CaCl_2$, 2; $MgCl_2$, 1 and KCl, 5) aerated with a mixture of 95% O_2/5% CO_2 and maintained at room temperature (22-25 °C). The tibial nerve was stimulated via a suction electrode at a frequency of 0.25 Hz with pulses of sufficient magnitude to produce a maximal twitch of the muscle (1 ms pulse duration; amplitude 0.5-2V). $MgCl_2$ (0.5-5 mM) or μ-Conotoxin GIIIB (2.5 μM; Alomone Labs) was added to the bathing medium to prevent muscle twitching during recordings and to reduce the size of the EPP to allow any enhancement to be observed. Tetraethylammonium applied in the presence of the BKCa channel blocker iberiotoxin (25-125 nM) and at low concentrations (0.05-0.5mM), which block Kv3 subunit containing channels specifically, did not affect muscle fibre resting potential but significantly increased the amplitude of EPPs ($p<0.05$, mean±SEM; 0.05 mM: 6.4±1.8 to 8.5±1.9mV (n=12); 0.1 mM: 5±0.9 to 7.7±1.2mV (n=6); 0.5 mM: 12.9±8.3 to 20.1±10.1mV (n=3)) Unexpectedly, blood depressing substance (BDS-I), a toxin selective for Kv3.4 subunits, had no effect at 0.05-

1μM. The combined presynaptic localisation of Kv3 subunits and enhancement of EPP amplitude indicate that Kv3 channels influence neurotransmitter release from presynaptic terminals. The channel at the motor nerve terminals may be unusual as it is insensitive to the Kv3.4 subunit specific toxin BDS even though Kv3.4 subunits are present.

We thank the British Heart Foundation for support (REB).

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C85

Substrate influences voltage-gated Na channel expression in strongly metastatic rat prostate cancer cell line

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During metastasis, cancer cells experience different substrates and undergo adhesion/detachement. We have shown previously that strongly metastatic prostate cancer (PCa) cells express voltage-gated Na channels (VGSCs) (e.g. Grimes et al.,1995). Blocking VGSCs with tetrodotoxin (TTX) suppresses cellular behaviours integral to the metastatic cascade, e.g. motility (Fraser et al.,2003) and secretory membrane activity (Mycielska et al.,2003). Here, we questioned whether VGSC activity could also be involved in cellular adhesion. The strongly metastatic rat PCa Mat-LyLu cells were grown on plain glass (PG) or gelatine-coated glass (GG) coverslips for up to 48 h with drugs added 3 h after plating. Voltage-gated currents were determined by whole-cell patch clamp recording. The holding potential was -100 mV. Maximal VGSC current densities were measured and analysed as means sem (n>20) and Student's t-test. VGSC expression was also studied by immunocyochemistry and confocal microscopy. Mat-LyLu cells grown on PG had VGSC current densities of 23.5 3.0 nA/pF (24 h) and 23.7 2.7 nA/pF (48 h). These were lower for cells grown on GG: 19.5 2.1 nA/pF (24 h) and 15.7 1.9 nA/pF (48 h). Resting membrane potential were depolarized ($p<0.001$): -48 2 mV (PG) and -41 2 mV (GG). Pre-incubation of cells grown on PG with TTX (1 uM) resulted in no change in current density ($p=026$). In contrast, Mat-LyLu cells grown on GG for 48 h in equimolar TTX had a significantly higher VGSC current density: 21.8 3.6 nA/pF ($p<0.02$ cf. untreated). Aconitine (100 uM), a VGSC 'opener' (Fraser et al.,2003) applied similarly had the opposite effect, giving the following VGSC current densities: 10.1 2.3 nA/pF (PG) and 3.7 0.6 nA/pF (GG). Similar changes were seen by immunocytochemistry. In conclusion, (1) substrate does influence VGSC expression/activity in Mat-LyLu cells; (2) forcing the cells to adhere more strongly (GG vs. PG) down-regulates VGSC expression; and (3) this change is controlled by VGSC activity. This study provides further evidence that VGSC upregulation could play a significant role in PCa metastasis directly by enhancing cellular activity as well as indirectly by modulating cellular signalling (e.g. Brackenbury & Djamgoz, 2003).

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This research was supported by the Pro Cancer Research Fund.

C86

cDNA microarray analysis of ion channel expression profiles in mature and proliferating human retinal pigment epithelium

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The retinal pigment epithelium (RPE) is a monolayer of cuboidal cells that lies in close association with the rod and cone photoreceptors and is thought to play an important role in setting up ion gradients within the interphotoreceptor matrix. Although electrophysiological recordings have permitted a rudimentary analysis of ion channel classes contributing to ionic movements across the RPE, little is known about the channel subtypes expressed in this tissue. In the present study we have used cDNA microarray technology to provide a more comprehensive overview of ion channel expression in both mature and proliferating human RPE cells.Total RNA was isolated from ARPE-19 cells after 1-mo (mature) or 4-d (proliferating) in culture. RNA was reversed transcribed and hybridised to cDNA microarrays containing 12,000 expressed human sequences (Agilent, human 1). Fluorescent labelling was performed with a 3DNA SubMicro kit (Genisphere) and spot intensities were measured using ScanAlyze (Stanford Computer Graphics Laboratory Freeware). Ion channel data was extracted from the microarrays using VectorXpression software (Informax) in conjunction with a purpose-built database (Microsoft Excel) comprising Genbank accession and Unigene numbers for all known α subunits of human ion channel genes.The ion channel database contained 252 ion channels, of which 137 were represented on the human microarrays. Based on replicate arrays, the same group of ion channels were found to be highly expressed in both mature and proliferating RPE. For example, six channels were found to be among the most highly expressed in both groups. Of these, an inward rectifier potassium channel (KCNJ13) and a chloride channel (CLCN1) have been previously characterised in RPE. The remaining four channels, a gap junction channel protein (GJA1), a gamma-aminobutyric acid receptor (GABAR1), a polycystic kidney disease cation channel (PKD2) and a voltage dependent anion channel (VDAC1) have not been previously identified. This study has demonstrated the use of cDNA microarrays for the rapid profiling of ion channel subtype expression in human RPE cells. Evidence has been obtained for the presence of previously uncharacterised channels and their functional significance now warrants further investigation. Overall, mature and proliferating RPE cells appear to display similar ion channel expression profiles.

We thank the Northern Ireland HPSS for financial support.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Sensing voltage in mammalian hyperpolarisation-activated HCN channels

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As observed for depolarisation-activated cation channels, the fourth transmembrane segment (S4) in hyperpolarisation-activated HCN channels displays a distinct stripe of positively charged residues. These positively charged residues are believed to form a charged peptide particle that senses changes in membrane voltage, leading to channel gating. The voltage-sensing mechanisms of S4 in depolarisation-activated cation channels have been extensively studied (Bezanilla, 2000; Cohen et al., 2003). However, mechanistic studies on the S4 voltage-sensor of hyperpolarisation-activated cation channels have only recently started to be defined. To address this lack of definition, the substituted-cysteine accessibility method was used to topologically map residue positions along the HCN1 S4 according to their accessibility to the membrane impermeant, cysteine(Cys)-specific reactive reagent [2-(trimethylammonium) ethyl] methane thiosulphonate (MTSET). An MTSET-resistant HCN channel background (HCN-R) was created by removing endogenous MTSET-reactive Cys residues. Consequently, only specified, substituted-Cys in the HCN-R background would be reported by MTSET reactivity. The resulting pattern of MTSET reactivity for the substituted-Cys revealed distinct environmental changes around the S4 of the HCN channel during membrane hyperpolarisation and depolarisation (i.e. during channel gating and closing). Based on these data, existing models of voltage-sensing movements of the S4 (primarily derived from studies on depolarisation-activated cation channels) do not comply with the mammalian hyperpolarisation-activated HCN channels. New models were explored to reconcile the observed MTSET accessibility of HCN Cys-substituted residues in this study, providing pertinent voltage-sensing mechanisms in these channels.

Bezanilla F, *Physiol. Rev.* **80**, 555-592.

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DCB was supported by the Wellcome Trust & the American Heart Association.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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ENZYMATIC BIOTINYLATION OF WILD-TYPE AND MUTANT CFTR CHANNELS

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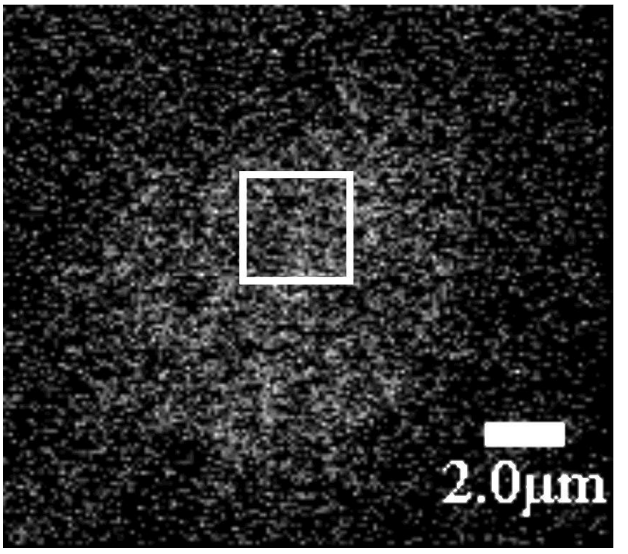
To study cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels at the cell surface we have engineered a target sequence for specific biotinylation into the fourth extracellular loop and stably expressed the mutant (CFTR-biotintag) in baby hamster kidney cells. Inserting the biotin acceptor did not compromise CFTR protein expression according to Western blots and had little effect on its channel function as assessed by iodide efflux assays and single channel recordings from excised, inside-out membrane patches. Channels were biotinylated *in situ* by exposing live cells to BirA, a biotin ligase that was cloned from *Escherichia coli*, expressed as a GST-BirA fusion protein, and added to extracellular solution containing ATP and biotin. Enzymatic biotinylation was used to purify CFTR protein on streptavidin beads. The yield of CFTR captured from cell lysates by this method was comparable to those obtained using immunopurification or Ni^{2+}-chelate affinity chromatography of polyhistidine-tagged (10x histidine) CFTR. After incubation with BirA, subsequent exposure to a fluorescent streptavidin conjugate produced strong plasma membrane labelling of cells expressing CFTR-biotintag, but negligable staining of cells expressing wild-type CFTR or untransfected control cells. Fluorescence was not detected at the surface of cells expressing ΔF508 CFTR-biotintag, although immature protein lacking complex glycosylation was demonstrated in Western blots, consistent with defective folding and retention of this disease-associated mutant in the endoplasmic reticulum. A time series of confocal fluorescence images was used to analyze the lateral mobility of wild-type channels on the cell surface by image correlation spectroscopy (Fig. 1*A*). The temporal autocorrelation function r (0,0,τ) was calculated (Fig. 1*B*) and fitted to a two dimensional diffusion model. This yielded a diffusion coefficient of $2.0 \pm 0.24 \times 10^{-11}$ cm^2 sec^{-1} (mean ± s.e., n = 20 cells) and immobile fraction that ranged between 0 and 43%. Similar mobilities were observed at 23 °C and 37 °C. Slow diffusion of CFTR channels in the plane of the membrane suggests their mobility may be impeded by interactions with other proteins. The results suggest that enzymatic biotinylation will be useful for purifying CFTR and studying its lateral diffusion at the cell surface.



Supported by the Canadian Institutes of Health Research, Canadian Cystic Fibrosis Foundation and Natural Sciences and Engineering Research Council (Canada) .