O20

Voltage-gated potassium channels in macrophages. A journey from proliferation to activation

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Potassium channels (KCh) have an important role on a number of physiological functions in mammalian cells. KCh are responsible for membrane excitability, secretion, activation and cell volume control among others (Felipe *et al.* 1993; Hille, 2001). Here we have studied the voltage-gated potassium channels and their regulatory subunits in bone marrow-derived macrophages (BMDM) to analyse their role in proliferation and activation. Macrophages originate from undifferentiated stem cells in the bone marrow and through the blood reach the different tissues and in most cases they undergo apoptosis. In response to the macrophage-colony stimulating factor (MCSF), macrophages are able to proliferate (Soler *et al.* 2001*a*). However, to carry out their functional activities, they must become activated. Lipopolysaccharide (LPS) is an activator of macrophages and it is also a potent apoptotic agent (Soler *et al.* 2001*b*).

We used BMDM isolated from mice as previously described (Soler *et al.* 2001*a*). Patch-clamp studies demonstrate that cells expressed outward delayed-rectifier and an inward rectifier K^+ currents. Pharmacological studies indicated that the outward current was generated by Kv1.3. In addition, Kir 2.1 was responsible for the inward rectifier K^+ current. Macrophages also express gene products from Kvβ1 and Kvβ2 modulatory subunits. MCSF-dependent proliferation induces both KCh and Kvβ auxiliary subunits in a time-dependent manner. These results were supported by an increase in KCh gene expression. When macrophages were activated with LPS, which stops proliferation, the KCh activity changed. While Kv1.3 further increased, Kir2.1 was down-regulated. Kvβ1 subunit gene products were differentially expressed and the Kvβ2.1 was up-regulated suggesting an increase in the amount and stability of the α/β membrane complex after LPS activation.

Our results describe for the first time the KCh and the Kvβ regulatory subunits present in BMDM. Proliferation is related to an overall increase in KCh activity and expression. However, activation induces a selective KCh differential regulation that could play a critical role to reach the immunitary response against infection.

Hille B (2001). *Ion Channels of Excitable Membranes*. Sinauer Associates, Sunderland, MA.

Felipe A *et al.* (1993). *Am J Physiol* **265**, C1230–1238.

Soler C *et al.* (2001*a*). *FASEB J* **15**, 1979–1988.

Soler C *et al.* (2001*b*). *J Biol Chem* **276**, 30043–30049.

This work was supported by MCYT, Spain (BFI2002-00764) and the University of Barcelona.

All procedures accord with current National and local guidelines.

O22

Glucose activates the volume-sensitive anion channel in rat pancreatic β-cells

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Glucose-stimulated insulin release depends on depolarisation of the β-cell plasma membrane and the generation of electrical activity. Depolarisation is thought to involve inhibition of K_{ATP} channels. However, we have recently reported that addition of a sub-stimulatory concentration of glucose (5 mM) was sufficient to completely inhibit K_{ATP} channel activity, and raising the glucose concentration to stimulatory levels (8–20 mM) had no further effect (Best, 2002*a*). Furthermore, glucose was able to evoke electrical activity in β-cells when K_{ATP} channel activity was completely inhibited by 0.5 mM tolbutamide (Best, 2002*a*). These findings indicate the existence of another ionic mechanism regulated by changes in glucose concentration.

Pancreatic β-cells were prepared from rats (humanely killed) and cultured for 1–14 days. Cell-attached channel recordings were made with a CsCl-rich pipette solution and diazoxide in the bath solution to clamp the membrane potential close to E_K. In 20–40 % of patches, an anion-selective channel of approximately 200 pS could be recorded, which conducted an inward current at a pipette potential of 0 mV (Best, 2002*b*). With 142 mM Cl^- in the pipette solution, current reversal occurred at a pipette potential of −52 mV. Channel activity was voltage dependent with a peak at a pipette potential of approximately −60 mV. The channel was activated by a rise in glucose concentration over the range 4–20 mM with a corresponding range of open probability from 0.07 to 0.86. Channel amplitude was not affected by altered glucose concentration. The channel was also activated by methylglyoxal, possibly due to its metabolism to D-lactate, but not by the non-metabolizable glucose analogue 3-O-methyl glucose. The channel was activated by hypotonic cell swelling and was sensitive to inhibition by the anion channel blockers 4,4′-dithiocyanatostilbene-2,2′-disulphonic acid, 5-nitro-2-(3-phenylpropylamino) benzoic acid, 4-hydroxytamoxifen and DCPIB (4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid).

It is suggested that the channel is the volume-sensitive anion channel previously described in insulin-secreting cells and that activation of this channel by glucose is the principal mechanism leading to depolarisation of the plasma membrane and hence electrical and secretory activity. The K_{ATP} channel could be important in hyperpolarizing the β-cell, thus preventing insulin release, during hypoglycaemia.

Best L *et al.* (1997). *Exp Physiol* **82**, 957–966.

Best L (2002*a*). *J Membr Biol* **185**, 193–200.

Best L (2002*b*). *Pflugers Arch* **445**, 97–104.

I should like to thank the NHS Executive North West for financial assistance.

All procedures accord with current UK legislation.

O23

Inhibition of Kv3 channels by peptidic toxins (BDS) from sea anemone

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A number of naturally occurring toxins have provided electrophysiologists with useful pharmacological tools for the study of ion channels. Many bind with high affinity and specificity to different Kvα subunits; some even distinguish between different members of the same Kv subfamily. Diochot *et al.* (1998) reported that the toxin, blood depressing substance (BDS), isolated from *Anemonia sulcata*, selectively blocked the rapidly inactivating Kv3.4 channel with an IC_{50} ~50 nM. Here we show BDS also inhibits Kv3.1 and Kv3.2 channels expressed in mammalian cell lines.

Recordings were made from HEK293 and B82 cells using whole cell voltage-clamp techniques. External and internal (pipette) solutions were based on Korngreen & Sakmann (2000). Voltage-gated K^+ currents were evoked by stepping the cells from a holding potential (V_H) of −80 mV to a variety of test potentials.

Both BDS-I and BDS-II (50–1000 nM) inhibited Kv3.1 currents in a concentration-dependent manner (Fig. 1). A single concentration of 500 nM was used in subsequent experiments as it resulted in approximately 50 % block – BDS-I: 48.1 ± 4.5 % (mean ± S.E.M., $n = 5$), BDS-II: 51.3 ± 5.4 % ($n = 4$).

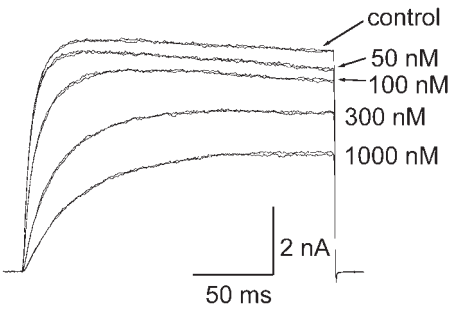

Figure 1. Kv3.1 current recordings (B82 cells) at a test potential of +40 mV in control and cumulative presence of BDS-II.

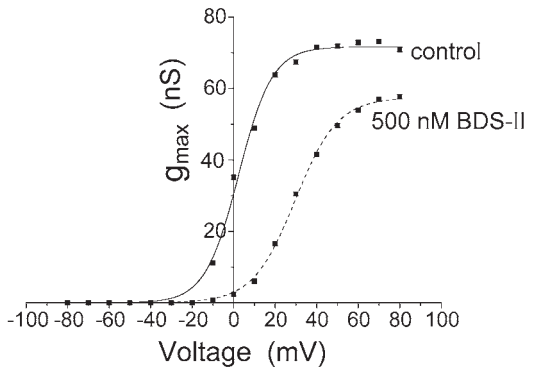

Figure 2. Conductance plots of Kv3.2 currents (HEK293 cells) in the absence and presence of 500 nM BDS-II.

Inhibition by BDS-I and -II caused a dramatic slowing in the rate of current activation (5- to 8-fold increase) at +40 mV with a positive shift in the activation threshold (~−20 mV, from approximately −10 to +10 mV, $n = 4$), suggesting these are 'gating modifier' toxins (e.g. Swartz & MacKinnon, 1997) (Fig. 2). Block did not require open channels, but was voltage dependent. With increasingly positive test potentials, the amount of block significantly reduced: e.g. +10 mV = 76.7 ± 2.7 % ($n = 4$), +80 mV = 21.0 ± 2.8 % ($n = 4$). The rate of Kv3 channel block was faster with BDS-II than BDS-I.

An endogenous current present in HEK 293 cells that were used to transiently express Kv3.2 channels was also blocked in a qualitatively similar manner by BDS. Antibody staining of these cells (Ruth Brooke, personal communication) suggests that Kv3.3 is present endogenously.

These data indicate that BDS-I and BDS-II are not selective blockers for the Kv3.4 channel.

Diochot S *et al.* (1998). *J Biol Chem* **273**, 6744–6749.

Korngreen A & Sakmann B (2000). *J Physiol* **525**, 621–639.

Swartz KJ & MacKinnon R (1997). *Neuron* **18**, 665–673.

This work is supported by the MRC.

O24

External acidosis abolishes the difference in response to ATP between large and small rat pulmonary artery smooth muscle cells

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The purine ATP can cause desensitizing contractions that may play a modulatory role in a variety of pathophysiological processes such as hypoxic pulmonary vasoconstriction and pulmonary hypertension (McCormack *et al.* 1993).

Isometric contractions were measured, as previously described (Vandier *et al.* 1997) in large (*ca* 700 μm e.d.) and small (*ca* 200 μm e.d.) intrapulmonary arteries (PA) isolated from rats killed with sodium pentobarbital (60 mg kg^{-1} I.P.) as approved by the Ministère Français de l'Agriculture. Before each ATP challenge, L-NAME (10^{-5} M) was added into each bath. Changes of $[Ca^{2+}]_i$ in individual arterial myocytes from large and small PA were recorded using the $[Ca^{2+}]_i$ sensitive fluorophore fura-2 and the fluorescence ratio (345/380) was calculated. Data are expressed as means ± S.E.M. and significance ($P < 0.05$) derived with ANOVA and Student's unpaired *t* tests.

At pH 7.4, the peak amplitudes of ATP (300 μM) contractions were not significantly different between small and large PA rings and nor was the increase of $[Ca^{2+}]_i$ ratio induced by ATP in isolated cells from small or large PA. Four successive applications of ATP (40 min apart) revealed a failure to resensitize the ATP response only in large PA rings, in which the peak amplitude of the response decreased, sequentially down to 39.4 ± 8.0 % of the first peak amplitude contraction ($n = 6$). This failure to resensitize was also observed in single cells isolated only from large PA and, in nine cells, the $[Ca^{2+}]_i$ ratio was decreased down to 37.2 ± 5.5 % of the initial peak response. External acidosis (pH 6.8) significantly decreased the amplitude of the first ATP contraction only in large PA rings (from 38.5 ± 1.9 % K80, $n = 7$ to 15.3 ± 2.9 % K80, $n = 6$). Interestingly, acidosis induced no significant change of resting $[Ca^{2+}]_i$ ratio in isolated cells from both PA populations. In the continued presence of external acidosis, successive ATP responses were now not significantly

different from the initial response both in large PA rings and in single cells from large PA. Furthermore, the amplitude of ATP contraction (% K80) in acidosis was not different between large and small PA rings.

The present study provides initial evidence of ATP receptor sensitization, which differs along the pulmonary artery tree. This phenomenon was modified with external acidosis suggesting an important physiological role of the desensitization–resensitization process in the regulation of pulmonary circulation.

McCormack DG *et al.* (1993). *Pulm Pharmacol* **6**, 97–108.

Vandier C *et al.* (1997). *Am J Physiol* **273**, H1075–H1081.

This work was supported by grant from Wellcome Trust and l'Agence De l'Environnement et de la Maitrise d'Energie.

All procedures accord with current National and local guidelines.

O25

Small-conductance calcium-activated potassium channels (SK3) play an inhibitory role in urinary bladder contractility

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The urinary bladder generates spontaneous contractions that are triggered by action potentials. Pharmacological evidence suggests that the after-hyperpolarization phase of these action potentials is mediated by apamin-sensitive small conductance K_{Ca} (SK) channels. The goal of this study was to determine the role of one of the known SK channel subtypes, SK3, in controlling the function of urinary bladder smooth muscle (UBSM).

We utilized transgenic mice targeted with a tetracycline-based genetic switch to control the expression of the SK3 gene. Homozygous targeted animals (SK3 T/T) over-express SK3 channels, while dietary treatment with doxycycline (DOX) suppresses SK3 expression below the levels of wild-type animals. We performed immunofluorescence microscopy to visualize the location of SK3 protein in the bladder wall of wild-type, SK3 T/T, and DOX-treated animals. To obtain the baldders, the animals were humanely killed.

SK3 channel expression was found in the urothelium and the UBSM layers of the bladder wall but no SK3 protein was seen in the intrinsic nerve plexus of the bladder. SK3 immunoreactivity was greatly increased in SK3 T/T animals, consistent with SK3-overexpression. DOX-treatment suppressed the level of SK3 immunoreactivity, consistent with suppression of SK3 expression. Electrophysiological recordings of SK currents in isolated UBSM cells revealed a threefold increase in SK current density in SK3 T/T myocytes. We characterized urodynamic performance in conscious mice instrumented with bladder catheters. The catheters were implanted under general anaesthesia with isoflurane (1–3%, inhaled). SK3 overexpressors had a dramatic increase in bladder capacity compared to wild-type animals (302 ± 41 μl *vs.* 140 ± 23 μl, mean ± S.E.M., $n = 7$, $P < 0.05$, Student's unpaired t test). Suppression of SK3 channel expression with DOX did not reverse this increase in bladder capacity (393 ± 41 μl), suggesting long-term structural remodelling of the bladder associated with SK3 over-expression. Suppression of SK3 channels with DOX did, however, induce bladder overactivity as indicated by a substantial (7-fold) increase in the number of non-voiding contractions per cystometrogram. In isolated strips of UBSM, spontaneous contraction frequency was elevated in strips from SK3 suppressed animals (DOX-treated). The SK channel blocker apamin increased contraction frequency and amplitude in strips from wild-type, SK3 T/T, and DOX-treated animals, but the effect was most pronounced in the SK3 overexpressors.

All together, these data suggest that SK3 channels inhibit urinary bladder contractility, and that both urothelial and smooth muscle SK channels may regulate bladder function.

This work was supported by NIH DK53832.

All procedures accord with current National guidelines.

P1

Random insertions of GFP analogues into the *hslo* channel

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In the past, analogues of green fluorescent protein (GFP) have been inserted randomly into several proteins using the Tn5 transposon (Sheridan *et al.* 2002). We have used this technique to randomly insert cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) sequence into multiple sites of the large conductance voltage- and calcium-dependent *hslo* channel (see Fig. 1). We have generated a library of 56 *hslo* fusion proteins. Coming next will be tests for function of these constructs.

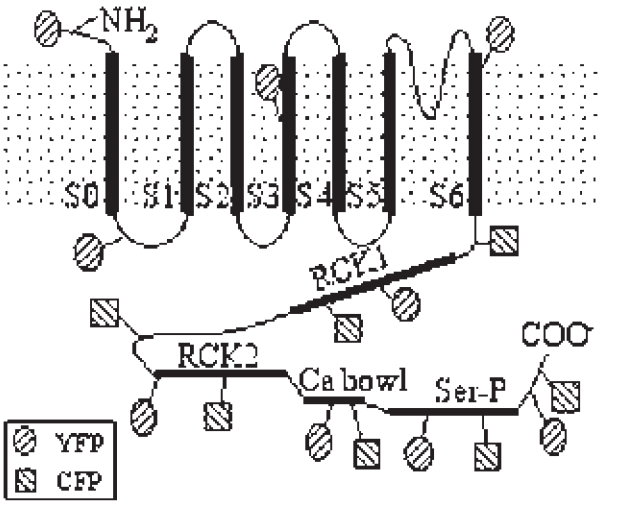

Figure 1. Insertions of CFP or YFP into the *hslo* channel.

Sheridan DL *et al.* (2002). *BMC Neuroscience* **3**, 7.

We thank Kate Klemic for helpful suggestions, and Klara Jacobsdottir for technical support.

P2

Oocytes and eggs of *Xenopus laevis* differ in the properties of calcium-dependent chloride channels

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Full-grown immature oocytes of *Xenopus laevis* express in their membrane a great number and variety of ion channels, including Ca^{2+}-dependent Cl^- channels (CaCCs). The aim of this work was to determine the properties of CaCCs in immature and mature oocytes, since heterogeneity of CaCCs has been proposed in immature oocytes (Boton *et al.* 1989; Kuruma & Hartzell, 1999).

To evoke Ca^{2+}-dependent Cl^- currents ($I_{Cl(Ca)}$) in immature oocytes, cells were permeabilized to Ca^{2+} with the ionophore A-23187 (2 μM, 15 min incubation in a Ca^{2+}-free Ringer solution) and challenged with 32 s pulses of Ringer solution containing 10 mM Ca^{2+}. At a holding potential of −60 mV, the elicited $I_{Cl(Ca)}$ showed two components: (i) a fast peak, which was almost fully blocked after adding to the Ringer solution either 100 μM niflumic acid (NFA), flufenamic acid (FFA), 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) or 1 mM 9-anthracene carboxylic acid (9-AC), and (ii) a slower one, much less sensitive to these blockers (the percentages of blockage were 29 ± 16 % (mean ± S.E.M.), n = 5; 77 ± 2 %, n = 3; and 50 ± 10 %, n = 3, for 100 μM NFA, FFA or NPPB, respectively; and less than 10 % for 1 mM 9-AC, n = 3). Eggs were obtained by incubating oocytes with progesterone (10 μM) for 12 h. The egg activation potential (AP) was evoked by either mechanical stimulation or by incubation with A-23187 (2 μM). The *I–V* relationship of the AP current showed a reversal potential of +30 ± 1 mV (n = 20), close to the E_{rev} for Cl^- (eggs were recorded in 10 % Ringer solution), indicating that it is due to CaCCs. Furthermore, replacement of Cl^- by other anions markedly affected the *I–V* curve. Neither 100 μM NFA, FFA or NPPB, nor 1 mM 9-AC blocked the AP, although it could be fully blocked by 1 mM DIDS. In fact, in eggs, $I_{Cl(Ca)}$ was potentiated by NFA, FFA and 9-AC at potentials more negative than −60 mV and was only moderately reduced at positive potentials. The mean open time of oocyte or egg CaCCs was assessed by voltage jumps. In immature oocytes, current relaxation curves of the slow component of the $I_{Cl(Ca)}$ could be fitted to a single exponential function, but two exponentials were required for egg $I_{Cl(Ca)}$ relaxation. In eggs, the values of the first component were similar to those obtained for immature oocytes (at −60 mV, τ = 21 ± 1 ms, n = 15, in oocytes, *versus* τ_1 = 23 ± 2 ms, n = 10, in eggs), but the second component was much slower (τ_2 = 125 ± 9 ms, n = 10).

These results suggest that either there are different subtypes of CaCCs in oocytes and eggs or, alternatively, they are differently regulated at these stages.

Boton R *et al.* (1989). *J Physiol* **408**, 511–534.

Kuruma A & Hartzell HC, (1999). *Am J Physiol* **276**, C161–175.

This work was supported by grant BFI2001-0756 from MCYT.

All procedures accord with current National guidelines.

P3

Changes in membrane conductance and capacitance induced by activation of *Xenopus laevis* eggs

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The activation potential (AP), like the fertilization potential, is a single active response that can be evoked in mature oocytes by increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The initial rise in $[Ca^{2+}]_i$, by Ca^{2+} entry, is further enhanced by a positive-feedback mechanism of Ca^{2+} release from internal stores. This results in a wave of high $[Ca^{2+}]_i$ travelling across the cell, which generates: (i) a large Ca^{2+}-dependent Cl^- current ($I_{Cl(Ca)}$), responsible for the AP, (ii) the exit from meiotic arrest, and (iii) the massive exocitosis of cortical granules (Evans & Florman, 2002). The aim of this work was to determine, simultaneously, the changes in membrane conductance (g_m) and capacitance (C_m) that follow egg activation, since fusion of a large number of cortical granules to the egg membrane and the subsequent membrane retrieval might affect the electrophysiological properties of the cell.

Eggs were obtained by incubating oocytes, isolated from a small piece of ovary, with progesterone (10 μM) for 12 h. Variations in g_m and C_m were simultaneously monitored, under voltage clamp conditions, by continuously applying, at 0.33 Hz, a brief (300 ms) ramp-pulse protocol, similar to that recently described by Schmitt & Koepsell (2002).

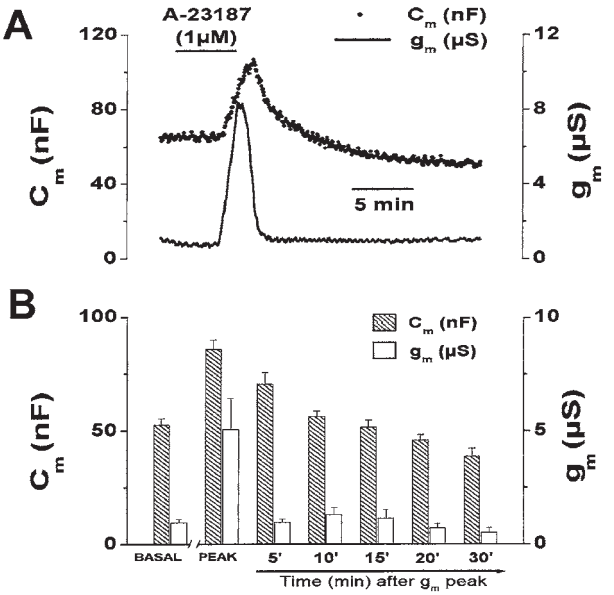

Figure 1. A, membrane conductance (g_m) and capacitance (C_m) changes elicited by A-23187 (1 μM) in an egg. B, histogram of g_m and C_m values at rest, peak and at several times after the g_m peak. Values are means ± S.E.M. of 5–11 eggs (except at 30 min where n = 3).

Superfusing the egg with the Ca^{2+} ionophore A-23187 (1 μM) elicited changes in g_m and C_m (see Fig. 1). g_m increased earlier and had a faster time course than C_m (Fig. 1A). So, the time to peak was 1.42 ± 0.23 min (mean ± S.E.M., n = 11) for g_m, *versus* 2.24 ± 0.39 min (same cells) for C_m. The duration of the peak (measured as the time required to return to basal values) was also shorter for g_m (4.09 ± 0.56 min, n = 11) than C_m (10.30 ± 1.25 min, same cells). At the peak, g_m values increased

about 5.3-fold over the basal ones while C_m increased 1.6-fold (Fig. 1B). Such an increase in C_m would be expected if 1.06×10^6 cortical granules of 1 μm in diameter were fused to the cell membrane by this time. Interestingly, both C_m and g_m values were smaller than basal ones over 20 min after g_m peak. This suggests that membrane retrieval after fusion of cortical granules is not a selective process, limited to endocytosis of the added membrane. This non-selective retrieval of cell membrane may contribute to the loss of ion channels and other molecules from the egg membrane after fertilization.

Evans JP & Florman H (2002). *Nature Cell Biol* **4**, S57–S63.

Schmitt BM & Koepsell H (2002). *Biophys J* **82**, 1345–1357.

This work was supported by grant BFI2001-0756 from MCYT.

All procedures accord with current National guidelines.

P4

Outwardly rectifying Cl^- currents activated in CAD cells in response to hypotonic stress

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The central nervous system catecholaminergic cell line CAD initially swells then undergoes a regulatory volume decrease (RVD) when exposed to a hypotonic environment (Harvey *et al.* 2002). RVD is usually accompanied by the loss of K^+ and Cl^- from the cell. A possible candidate for the anion exit pathway is the volume-regulated anion channel (VRAC) reported to be present in many cell types (Lang *et al.* 1998).

Using the whole-cell configuration of the patch-clamp technique, CAD cells exhibited reversible activation of an outwardly rectifying current in response to hypotonic stress. Data reported below were all obtained by 100 ms step depolarisations from −80 to +100 mV from a holding potential of −60 mV, and are expressed as mean ± S.E.M. current densities (pA pF^{-1}). Significant differences were determined using Student's paired *t* test or ANOVA followed by Tukey's HSD test.

Cells were bathed in isotonic bath solution containing (mM) NaCl 110, $CaCl_2$ 1, $MgCl_2$ 1, Hepes 10, adjusted with NaOH (pH7.3) and mannitol (300 mosmol $(kg\ H_2O)^{-1}$), and dialysed with a pipette solution containing (mM) NaCl 50, EGTA 1, $MgCl_2$ 1, Hepes 10, adjusted with NaOH (pH7.3) and mannitol (290 mosmol $(kg\ H_2O)^{-1}$). Under these conditions CAD cells displayed an outward current of 7.8 ± 1.9 pA pF^{-1} and inward current of −0.5 ± 0.5 pA pF^{-1} ($n = 13$). After a 5 min exposure to an iso-ionic hypotonic solution (230 mosmol $(kg\ H_2O)^{-1}$) the outward current increased to 40.6 ± 7.1 pA pF^{-1} and the inward current increased to −11.8 ± 2.7 pA pF^{-1} ($n = 13$, $P < 0.001$, Student's paired *t* test). The reversal potential (E_{rev}), −15.6 ± 1.6 mV ($n = 13$) was similar to the theoretical E_{rev} for a Cl^--selective conductance under these conditions.

The swelling-activated current was inhibited by the Cl^- channel antagonist 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS). Bath perfusion with 100 μM DIDS reduced the outward current from 50.3 ± 11.8 to 21.9 ± 4.4 pA pF^{-1} ($n = 4$, $P < 0.05$, ANOVA), but had no significant effects on the inward current. At positive potentials the channel block mediated by DIDS was dependent on the applied transmembrane potential. This inhibition was reversed to 43.2 ± 14.5 pA pF^{-1} ($n = 4$) following washout with hypotonic solution.

In conclusion, CAD cells possess swelling-activated currents that display properties consistent with those of the VRAC reported in other cell types. Since 100 μM DIDS also inhibits RVD in CAD cells (Harvey *et al.* 2002), these currents may represent the pathway for the extrusion of Cl^- during RVD.

Harvey V *et al.* (2002). *J Physiol* (in the Press).

Lang F *et al.* (1998). *Physiol Rev* **78**, 247–306.

P5

Plasma membrane voltage-dependent anion channel mediates antioestrogen-activated Maxi Cl^- current in mouse C1300 neuroblastoma cells

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The voltage-dependent anion-selective channel (VDAC) is a pore-forming protein abundant in the outer mitochondrial membrane. Its presence in extramitochondrial localizations remains controversial, although recently a plasma membrane localization has received support from the identification of a membrane leader sequence in VDAC (Buettner *et al.* 2000). The biophysical properties of VDAC resembled those of Maxi Cl^- channels found in the plasma membrane of a wide variety of cell types. Maxi Cl^- channels have typically been observed only in excised patches, suggesting their regulation by intracellular signals (Diaz *et al.* 2001). We have also observed that Maxi Cl^- channels could be reversibly activated by antioestrogens under whole-cell and cell-attached recording conditions (Hardy & Valverde, 1994; Diaz *et al.* 2001). However, the physiological relevance of these channels remains unknown.

In the present report we have identified in C1300 mouse neuroblastoma cells a plasma membrane isoform of VDAC (pl-VDAC) by RT-PCR using primers directed against the plasma membrane leader sequence of VDAC (Buettner *et al.* 2000). The use of a monoclonal antibody against the N-terminus of the mitochondrial VDAC (Calbiochem) allowed us to identify a band of ~31 kDa (expected size for VDAC) in C1300 membrane fractions and localized VDAC to the plasma membrane by immunofluorescence confocal microscopy. Moreover, VDAC co-localised with markers of membrane lipid rafts (cholera toxin β subunit) but not caveolin-1.

The relationship between VDAC and Maxi Cl^- currents activated by antioestrogens was investigated using plVDAC antisense oligonucleotides. Transient transfection of C1300 cells with an antisense directed against the membrane leader sequence of VDAC markedly reduced both the expression of VDAC and the Maxi Cl^- current activated by the antioestrogen toremifene. Whole-cell toremifene-activated Maxi Cl^- current density recorded in control untreated cells (195 ± 50 pA pF^{-1}), β-globin antisense-transfected cells was 220 ± 50 pA pF^{-1} (mean ± S.E.M.; $n = 7$) and 50 ± 13 pA pF^{-1} ($n = 11$) in pl-VDAC antisense transfected cells ($P = 0.05$, one-way ANOVA followed by Bonferroni post test).

In summary, our data provides strong support for the presence of VDAC protein in the plasma membrane, including specialized micro-domains such as lipid rafts, and suggests that VDAC is the molecular counterpart of the antioestrogen-activated Maxi Cl^- channel of C1300 neuroblastoma cells.

Buettner R *et al.* (2000). *Proc Natl Acad Sci USA* **97**, 3201–3206.

Diaz M *et al.* (2001). *J Physiol* **536**, 79–88.

Hardy SP & Valverde MA (1994). *FASEB J* **8**, 760–765.

This work was funded by the Human Frontiers Science Program.

P7

Identification of a binding site for the kinase-anchoring protein, AKAP79 on the murine inwardly rectifying potassium channel, Kir2.1

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Protein kinase A (PKA) is an important, broad-specificity kinase that is activated by the diffusible second messenger cAMP. Since PKA is capable of phosphorylating a broad range of target proteins, specificity in PKA signalling is achieved via A-kinase anchoring proteins (AKAPs) that segregate different pools of PKA with their appropriate substrate. Ion channels represent a diverse group of kinase substrates, and kinase targeting through association with AKAPs facilitates PKA-mediated phosphorylation and regulation of AMPA/kainate receptors, L-type calcium channels and calcium-activated and inwardly rectifying potassium channels (Fraser & Scott, 1999).

We have shown recently that cAMP-dependent modulation of whole-cell currents of the inwardly rectifying potassium channel, Kir2.1, requires the presence of the multivalent anchoring protein, AKAP79, and that Kir2.1 and AKAP79 exist together in a complex within intact cells (Dart & Leyland, 2001). Here, we extend this study by identifying a binding site of AKAP79 on the intracellular C-domain of Kir2.1.

To determine possible sites of interaction, glutathione-S-transferase (GST) fusion proteins of various regions of the C-terminal domain of Kir2.1 were screened for their ability to isolate AKAP79 from lysates of HEK293 cells transfected with cDNA encoding AKAP79 (a gift from Dr John Scott, Vollum Institute, Portland, OR, USA). A GST fusion protein of residues 182–282, GST-C(182–282) was found to isolate AKAP79 from HEK293 lysates, while GST alone and GST fusion proteins of other regions of the C-terminus failed to interact with AKAP79 ($n = 3$). Additional smaller fusion proteins made from the interacting region showed that GST-C(182–232) retained its ability to isolate AKAP79, while GST-C(233–282) did not ($n = 3$).

These data suggest that AKAP79 targets a region between residues 182 and 232 on the intracellular C-domain of Kir2.1, presumably to anchor PKA in close proximity to channel phosphorylation sites. Interestingly, this region of Kir2.1 contains a modified leucine zipper-like motif (a periodic repetition of hydrophobic residues at every seventh position) that has been shown to mediate AKAP binding to L-type calcium channels and to the KvLQT1 ion channel (Hulme *et al.* 2002; Marx *et al.* 2002). This motif may represent a conserved mechanism by which AKAPs interact with ion channels.

Dart C & Leyland ML (2001). *J Biol Chem* **276**, 20499–20505.

Fraser IDC & Scott JD, (1999). *Neuron* **19**, 185–196.

Hulme JT *et al.* (2002). *J Biol Chem* **277**, 4079–4087.

Marx SO *et al.* (2002). *Science* **295**, 496–499.

This work was supported by the MRC and the Royal Society.

P8

Functional and molecular characterization of TRPV4 cation channel in human bronchial epithelial 16HBE14o− cells

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Airway epithelial cells respond to changes in the osmolarity of airway surface liquid. In 16HBE14o− cells, a human bronchial epithelial (HBE) cell line, regulatory volume decrease (RVD) requires the activation of a Ca^{2+}-dependent potassium channel, triggered by the influx of Ca^{2+} via a gadolinium (Gd^{3+})-sensitive, swelling-activated cation channel (Fernández-Fernández *et al.* 2002) of unknown molecular identity. TRPV4, a member of the transient receptor potential family of ion channels, which is osmosensitive and inhibitable by Gd^{3+}, has been recently described (Strotman *et al.* 2000). The aim of the present work was to characterize the functional expression of TRPV4 channels in HBE cells.

Expression of TRPV4 in HBE cells was assessed using RT-PCR and Western-blot techniques. Full-length cDNA amplification indicated two isoforms of which one lacked exon 6, encoding part of the third ankyrin repeat and one protein kinase C phosphorylation site. Western-blot analysis using a polyclonal antibody against the C terminus of TRPV revealed a band of ~120 kDa.

The activity of TRPV4 channels in HBE cells was examined measuring intracellular Ca^{2+} with Fura-2 fluorescent dye. Changes in Ca^{2+} were measured as the ratio of emission fluorescence intensities obtained following excitation with 340 nm and 380 nm wavelengths in cells loaded with 2 μM Fura-2. Cells challenged with a 28 % hypotonic solution responded with a transient increase in fluorescence ratio from 0.35 ± 0.01 to 0.5 ± 0.03 ($n = 3$, means ± S.E.M.). The phorbol ester analogue, 4α-phorbol 12,13-didecanoate (4α-PDD, 1 μM), known to activate TRPV4 (Watanabe *et al.* 2002) induced a larger increase in the fluorescence ratio from 0.35 ± 0.01 to 1.18 ± 0.1 ($n = 3$). In the presence of 100 μM Gd^{3+}, the fluorescence ratio in response to hypotonic shock and 4α-PDD changed from 0.34 ± 0.02 to 0.37 ± 0.02 and from 0.4 ± 0.006 to 0.54 ± 0.02, respectively ($n = 4$). The electrophysiological activity of TRPV4 channels was also recorded in HBE cells using a NaCl-rich bathing solution containing 2 mM $CaCl_2$ and a Ca^{2+}-free intracellular *N*-methyl-D-glucamine chloride solution. A reversible increase in cationic current density from -3.21 ± 2.4 pA pF^{-1} (control) to -27.3 ± 10.6 pA pF^{-1} ($n = 4$) was recorded (at −120 mV) upon addition of 1 μM 4α-PDD.

Our data provide the first functional and molecular identification of endogenously expressed TRPV4 channels in human airway epithelia.

Fernández-Fernández JM *et al.* (2002). *Am J Physiol Cell Physiol* **283**, C1705–1714.

Strotman R *et al.* (2000). *Nat Cell Biol* **2**, 695–702.

Watanabe H *et al.* (2002). *J Biol Chem* **277**, 13569–13577.

This work was supported by Ministerio de Ciencia y Tecnolog'a (España) and Distinció de la Generalitat de Catalunya.

P9

The expression and specific localization of an inwardly rectifying K^+ channel, Kir5.1, in the spiral ligament of rat cochlea

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The cochlea of the inner ear is filled with two different types of fluid: a unique extracellular solution, endolymph, which has a positive potential of ~+80 mV and 150 mM K^+ ions, and a normal extracellular solution, perilymph. The specialized ionic composition and high potential in endolymph is essential for hearing. The circulation of K^+ ions from perilymph to endolymph through the cochlear lateral wall is crucially involved in establishment and maintenance of endolymph. We reported previously an inwardly rectifying K^+ channel, Kir4.1, expressed in stria vascularis in cochlea. The exact molecules responsible for the K^+ transport, however, have remained largely unknown.

Here we report that an inwardly rectifying K^+ channel, Kir5.1, is strongly expressed in the spiral ligament of the lateral wall. A specific antibody labelled Kir5.1 in type II and IV fibrocytes in spiral ligament that are exposed to the perilymph in scala tympani. On the other hand, no Kir5.1 immunoreactivity was detected in either type I or III fibrocytes of the ligament. Immuno-electron microscopic examination revealed that Kir5.1 is abundantly localized in the processes of the fibrocytes. Developmental expression of Kir5.1 was paralleled with those of Na^+,K^+-ATPase and Na^+–K^+–$2Cl^-$ cotransporters, the molecules important for K^+ transport in the ligament. Furthermore, Kir5.1 also occurs at the fibrocytes of suprastrial zone of the ligament and those of the spiral limbus that face to another perilymphatic space, the scala vestibuli.

These results suggest that the Kir5.1 channel plays a central role in recycling action and homeostasis of K^+ ions in cochlea that are indispensable for auditory function.

All procedures accord with current National guidelines.

P10

Insulin activates human large conductance Ca^{2+}-activated K^+ (BK) channels via a PI 3-kinase-independent pathway

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Growing evidence suggests that the endocrine hormones insulin and leptin are important modulators of neuronal function, with actions not directly associated with energy homeostasis. For example, leptin inhibits hippocampal neurons by phosphoinositide 3-kinase (PI3K)-driven activation of large conductance calcium-activated K^+ (BK) channels (Shanley *et al*, 2002). In this study we have used human embryonic kidney (HEK 293) cells stably transfected with the α (*hSlo*) subunit of the BK channel to examine the actions of insulin.

Cell-attached and inside-out patch clamp recordings were used to study single channel responses as described previously (Shanley *et al.* 2002). In all cell-attached recordings, a large conductance K^+ channel was evident ($n = 164$). In symmetrical 140 mM K^+ inside-out recordings, the channel displayed a linear *I–V* relationship (± 60 mV) with a slope of conductance 210 ± 8.7 pS (mean ± S.E.M., $n = 6$) and reversal potential close to 0 mV. Channel open probability (P_o) increased with membrane potential depolarisation or increasing $[Ca^{2+}]_i$, such that the $V_{0.5}$ values obtained in 1 μM and 10 μM Ca^{2+} were 37.29 ± 7.1 mV ($n = 6$) and 12.29 ± 4.9 mV ($n = 5$), respectively. Pipette application of insulin (1–50 nM) during cell attached recordings caused a rapid increase in channel activity ($n = 31/35$) with mean channel activity (NfP_o) increasing from 0.09 ± 0.02 (2–4 min) to 0.31 ± 0.09 ($n = 35$, $P < 0.05$, ANOVA) after 15 min. In contrast, control recordings displayed no change in channel activity with time ($n = 17$). As insulin and insulin-like growth factor-1 (IGF-1) receptors have a high sequence homology, a comparison of potencies of insulin and IGF-1 was used to determine the receptor identity. Thus, in the presence of insulin (10 nM), channel activity at 15 min was 2.87 ± 0.6 ($n = 8$), while NfP_o 15 min after application of IGF-1 (10 nM, $n = 5$) was 2.50 ± 0.41 (means normalised to control at 2–4 min; $P > 0.05$), suggesting the involvement of the IGF-1 receptors. It is well established that PI3K is a key enzyme activated downstream of IGF-1 receptors. However, incubation with LY294002 (10 μM) or wortmannin (10–50 nM) failed to prevent insulin activation of BK channels. Thus, the normalised NfP_o values obtained 15 min after insulin addition following exposure to LY294002 or wortmannin were 3.45 ± 0.8 ($n = 7$) and 2.6 ± 0.66 ($n = 7$), respectively compared to a control value of 3.81 ± 0.75 ($n = 35$).

These data indicate that insulin acting on IGF-1 receptors stimulates BK channels via a PI3K-independent pathway. This process may be a novel mechanism for regulating excitability.

Shanley LJ *et al.* (2002). *Nat Neurosci* **5**, 299–300.

This work is supported by the Wellcome Trust and Tenovus Scotland.

P13

Voltage-dependent potassium channels in human adipose cells

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Many hormonal effects in eukaryotic cells are produced through changes in ionic membrane permeabilities and subsequent changes in membrane potential. Although a great deal is known concerning white fat metabolism, little information about its electrophysiology is available. We developed an experimental model that allowed us to obtain mature white adipocytes from preadipocytes of epididymal tissue of rat (Ramírez-Ponce *et al.* 1996). In these cells we demonstrated the existence of voltage-dependent K^+ channels (Kv), using the whole-cell variant of the patch-clamp technique.

To date, there are no electrophysiological data referring to human white adipocytes, and only indirect studies have suggested the existence of ionic channels in these cells. In metabolic studies, it is common to find distinct properties and regulatory mechanisms between different cell models derived from diverse species and development stage. For this reason, it would be interesting to know if human adipocytes have ionic channels, and if these have the same properties as the channels of rat adipocytes. This fact will increase interest in the electrophysiology of white adipose tissue and its possible physiological importance.

In this study, the whole-cell voltage-clamp method was used to study the membrane electrical properties of human adipocytes

cells obtained by differentiation from precursors of human abdominal and mammary tissues. Subcutaneous and visceral samples of human fat were obtained from normal-weight subjects (< 27 kg m^{-2}) undergoing abdominal surgery or surgical mammary reduction. Patients with malignant or chronic inflammation diseases were excluded. The study was approved by the Ethics Committee of the Hospital Virgen Macarena de Sevilla. All differentiated cells exhibited outward currents with a sigmoidal activation kinetic. The outward currents showed activation thresholds between −20 and −30 mV and a slow inactivation. The ionic channels underlying the macroscopic current were highly selective for K^+. Their selectivity was typical for K^+ channels with relative permeabilities of $K^+ > NH_4^+ > Cs^+ > Na^+$. There was no evidence of any other type of voltage-gated channel. The potassium currents were blocked reversibly by tetraethylammonium and barium. The IC_{50} values and Hill coefficient of tetraethylammonium inhibition of $I_K V$ were 0.56 mM and 1.17 respectively. These results demonstrate that human adipose cells have voltage-dependent potassium currents.

Ramírez-Ponce MP *et al.* (1996). *Biochem Biophys Res Comm* **223**, 250–256.

All procedures accord with current local guidelines and the Declaration of Helsinki.

P14

Voltage-dependent potassium channels may modulate the adipogenesis in white adipocytes of rat: insulin effects

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Recently, there has been a dramatic increase in the incidence of obesity resulting from an excess of white adipose tissue. This obesity may occur as a result of the enlargement of existing adipocytes and/or as a consequence of an increase in new fat cells. Although multiple factors modulate proliferation and differentiation of adipocytes, it is known that some hormones and growth factors act via specific receptors to transduce external growth and differentiation signals through a cascade of intracellular events. Insulin is able to stimulate cell proliferation in white adipose tissue and increases the number of differentiated cells for most primary preadipocytes. Experiments with other cell types have implicated potassium channels as being essential for their proliferation and differentiation. It has been demonstrated that functional voltage-gated potassium channels may be necessary for the normal proliferation and differentiation of brown fat cells in culture and that the purinergic modulations of these potassium currents may be important for altering adipocyte growth and development.

In white adipocytes, obtained by culturing preadypocytes from epididymal tissue of rats that had been humanely killed, we demonstrated the existence of voltage-dependent K^+ channels (Kv) using the whole-cell variant of the patch-clamp technique (Ramírez-Ponce *et al.* 1996). In a previous study carried out by means of intracellular recording, we showed that insulin modulates K^+ conductances in white adipocytes (Ramírez-Ponce *et al.* 1991).

In this work we studied the potassium currents in white adipocytes obtained by culturing preadipocytes from rat epididymal tissue, both with insulin (WAi) and without insulin (WAo), in order to test the role of insulin in the development of voltage-gated potassium channels (Kv) during the adipogenesis. Occasionally, very small potassium currents (I_{K_V}) were present in preadipocytes; nevertheless these currents were measured in all differentiated cells (adipocytes). WAi exhibited greater macroscopic potassium currents than WAo with no apparent differences in kinetics or voltage dependence. The current density (pA μm^{-2}) calculated in WAi was higher than in WAo. Currents were blocked by millimolar concentrations of tetraethylammonium (TEA). The effect of insulin on adipogenesis, both with and without TEA, was analysed. Four days without insulin and three days with insulin were necessary to increase 2.5-fold the total number of cells in culture. Insulin increased the number of differentiated cells by 73.5 %. Cell proliferation and differentiation were inhibited by TEA. Proliferation was affected only by a high concentration of TEA. Inhibition of differentiation was dose dependent, with concentration for half-block similar to the IC_{50} values to block potassium channels. These results suggest that insulin increases the density of Kv and that these channels may be necessary for the normal growth of white adipocytes in culture.

Ramírez-Ponce MP *et al.* (1991). *Rev Esp Fisiol* **47**, 217–222.

Ramírez-Ponce MP *et al.* (1996). *Biochem Biophys Res Comm* **223**, 250–256.

All procedures accord with current national guidelines.