C45

Novel mutations of Kir2.1 underlying Andersen's syndrome are non-functional and have a dominant negative effect on the wild-type allele

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Andersen's syndrome is a rare disorder characterised by periodic paralysis, cardiac arrhythmias and dysmorphic features. It occurs either sporadically or as an autosomal dominant disease with highly variable traits among members of the same affected family. Recently, missense mutations of KCNJ2, the gene encoding the inward rectifier potassium channel Kir2.1, were identified in Andersen's syndrome (Plaster *et al.* 2001). To date twelve point mutations have been characterised, all involving residues that are highly conserved across all Kir subunits, and that lie in functionally important domains of the channel. Mutants that have been studied in heterologous expression fail to form functional channels when expressed alone, and cause variable degrees of dominant-negative effect when co-expressed with wild-type (wt) Kir2.1.

We have identified six additional mutations in British families with Andersen's syndrome (with informed consent), in which probands presented with periodic paralysis. The mutations occur in either the N-terminus (R67W, T75M, D78G, R82Q) or the C-terminus (L217P, G300D), but none affects either the transmembrane segments or the pore-lining loop. All but one (R67W) of these mutations are previously undescribed. We injected cRNA coding for wt and/or mutant Kir2.1 into *Xenopus laevis* oocytes obtained using standard methods, and recorded K^+ currents with the two electrode voltage-clamp technique. All four mutants studied (T75M, R82Q, L217P, G300D) were non-functional, and caused a complete dominant-negative suppression of channel function when co-expressed with wt. No significant functional differences were identified among the mutants.

Kir2.1 is expressed in heart, skeletal muscle and brain where it contributes to the repolarising phase of action potentials, and to stabilisation of the membrane resting potential. Reduction of Kir2.1 function is expected to increase membrane excitability, thus explaining the cardiac arrhythmias and periodic paralysis. The developmental characteristics of the Kir2.1 knockout mouse (narrowing of the maxilla and cleft of the secondary palate) are consistent with the dysmorphic features seen in many Andersen's syndrome patients, suggesting an additional role for this channel in musculoskeletal development.

Plaster NM *et al.* (2001). *Cell* **105**, 511–519.

All procedures accord with current UK legislation.

C46

The involvement of residues H98 and E70 in the block of the human two pore domain potassium channel, TASK-3, by zinc

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Zinc is essential for brain function. It is sequestered into and released from specialised 'zinc-containing' neurons. An increased zinc concentration in the extracellular solution has been shown to alter the activity of several membrane receptors and channels (e.g. Takeda, 2000). In the present study we have investigated the effects of extracellular zinc on TASK-1 and TASK-3 channels, acid-sensitive members of the 'background' two-pore domain potassium channel family.

Human TASK-1 (hTASK-1) and hTASK-3 were expressed in *Xenopus* oocytes as previously described (Clarke *et al.* 2000). Relatively large, outwardly rectifying currents were recorded in low external K^+ (1 mM) when voltage ramps from −120 to +80 mV were applied. These currents were blocked by acidification of the external solution. Full pH response curves show that hTASK-1 is more pH sensitive that hTASK-3 with pH IC_{50} values of 7.3 ± 0.08 and 6.6 ± 0.04, respectively (means ± S.E.M.).

Application of 100 μM zinc had no effect on hTASK-1 currents (-4.4 ± 6.5 %, $n = 10$), a small inhibitory effect on rat TASK-1 currents (17.1 ± 3.9 %, $n = 5$), but blocked hTASK-3 currents, in a voltage-independent manner, by 68.9 ± 5.2 % ($n = 9$). The zinc concentration–response curve for hTASK-3 had an IC_{50} of 19.8 ± 2.7 μM ($n = 6$). Mutation of histidine (H) 98 of hTASK-3 to alanine (A) by site-directed mutagenesis, which led to a loss of pH sensitivity of the channel, also reduced the zinc sensitivity of the channel (with an IC_{50} of 95.8 ± 14.1 μM, $n = 5$).

As the equivalent histidine residue is also present in hTASK-1 and as zinc sensitivity is not completely lost in mutant H98A of TASK-3, it appeared that another residue might also be involved in the blocking action of zinc. Previous work by Derst *et al.* (2002) has identified glutamic acid (E) 70 of TASK-3 as an important determinant of sensitivity to extracellular divalent cations. TASK-1 does not have the equivalent residue in this position. Mutation of hTASK-3 E70 to lysine (K) by site-directed mutagenesis also led to a reduction of zinc block, with an IC_{50} of 104.8 ± 24.3 μM.

In conclusion, our data suggest that both residues H98, predicted to lie at the mouth of the first pore region and E70, which is within the large M1-P1 loop, are involved in the selective block by zinc of human TASK-3 channels.

Clarke CE *et al.* (2000). *J Physiol* **523**, 697–703.

Derst C *et al.* (2002). *Pflügers Arch* **443**, S340.

Takeda A (2000). *Brain Res Rev* **34**, 137–148.

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All procedures accord with current UK legislation.

C47

Modulation of the voltage-gated potassium (Kv) channel currents by intracellular Mg^{2+} in rat aortic smooth muscle cells (RASMCs)

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Potassium channels play a key role in the regulation of excitation–contraction coupling in vascular SMCs and Kv channels, being ubiquitous in the vasculature, are important contributors to the regulation of vascular tone. Intracellular mechanisms controlling function of Kv channels in arteries are poorly understood. We have previously found that the replacement of 5 mM MgATP with 5 mM $MgCl_2$ in the pipette solution caused a significant negative shift in the Kv channel current (I_{Kv}) availability in RASMCs (Tammaro *et al.* 2001). Gelband *et al.* (1993) showed previously that increase in intracellular divalent cation concentration significantly suppressed I_{Kv} in isolated SMCs. Since ATP is a potent chelator of Mg^{2+}, a possibility of the direct effect of intracellular Mg^{2+} on I_{Kv} was further investigated in aortic SMCs isolated from humanely killed male Wistar rats.

Kv channel currents were isolated using paxilline (1 μM) and glibenclamide (10 μM) in the external solution to block Ca^{2+}-activated and ATP-sensitive K^+ currents, respectively. Pipette solution contained (mM): 110 KCl, 10 NaCl, 10 Hepes, 10 EGTA and 0.5 $CaCl_2$; pH 7.2. Intracellular [Mg^{2+}] was altered by adding $MgCl_2$. Increasing the pipette concentration of $MgCl_2$ from 0.5 mM (used as control) to 5 mM did not significantly affect either I_{Kv} steady-state activation (calculated from *I–V* relationships) or the whole-cell maximum conductance (0.077 ± 0.009 pS pF^{-1}, $n = 12$ and 0.074 ± 0.007 pS pF^{-1}, $n = 15$; mean ± S.E.M.). However, I_{Kv} availability, measured with 10 s conditioning pre-pulses, was significantly shifted by ~9 mV ($P < 0.013$, unpaired *t* test) with $V_{0.5} = -45.4 \pm 1.7$ mV ($n = 6$) and $V_{0.5} = -53.7 \pm 1.9$ mV ($n = 11$) for 0.5 and 5 mM $MgCl_2$, respectively. Increase in the pipette concentration of $MgCl_2$ to 10 mM produced further shifts in I_{Kv} availability ($V_{0.5} = 61.7 \pm 5.7$ mV, $n = 6$). Although steady-state activation was not affected ($P > 0.05$), whole-cell maximum conductance was decreased by 47% to 0.04 ± 0.004 ($n = 6$, $P < 0.002$) by 10 mM $MgCl_2$. Perfusion of RASMCs with pipette solution containing 0.5 mM $MgCl_2$ with pH adjusted to 6.2 did not significantly affect either voltage dependency I_{Kv} activation and inactivation or the maximum conductance.

A selective effect of intracellular Mg^{2+} on I_{Kv} inactivation and not activation dependencies and the lack of the effect of increased intracellular concentration of protons on I_{Kv} does not support direct interaction between Mg^{2+} and fixed surface charges near the internal channel mouth, and could indicate a novel mechanism of the modulation of Kv channels by physiological concentrations of intracellular Mg^{2+} in VSMCs.

Gelband CH *et al.* (1993). *Circ Res* **73**, 24–34.

Tammaro P *et al.* (2001). *Biophys J* **80**, 440a.

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PC68

Regulation of homomeric Kir3.1F137S channels: a comparison with the cloned neuronal channel, Kir3.1+3.2A

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G protein-gated inwardly rectifying K^+ channels (Kir3.x family) are predominantly expressed in neuronal and atrial tissue and characteristically are activated directly by $\beta\gamma$ dimers released following stimulation of $G_{i/o}$- but not G_s-coupled receptors.

We have previously demonstrated that stimulation of a number of $G_{q/11}$-coupled receptors results in biphasic modulation of the cloned neuronal channel, Kir3.1+3.2A: an initial enhancement of currents is followed by a profound and long-lasting inhibition (Leaney *et al.* 2001).

Kir3.1 expressed on its own does not form functional channels, although introduction of a point mutation in the pore region, F137S, results in functional homomeric channels (Chan *et al.* 1996). We investigated whether such channels (herein designated as Kir3.1F137S) exhibited regulation by $G_{i/o}$- and $G_{q/11}$-coupled receptors in a similar fashion to Kir3.1+3.2A channels.

Whole-cell patch clamp was used to record membrane currents under symmetrical K^+ conditions (~140 mM K^+, ATP/GTP-containing pipette solution) in HEK293 cells transiently expressing the appropriate channel subunits and receptors. Data are presented as means ± S.E.M. and one-way ANOVA was used to test for statistical significance.

Similarly to Kir3.1+3.2A channels, Kir3.1F137S currents were reversibly inhibited by external barium and enhanced ~ 4-fold by overexpression of $G\beta1\gamma2$. Furthermore, stimulation of transiently expressed $G_{i/o}$-coupled A_1 adenosine receptors by 5′-*N*-ethylcarboxyamidoadenosine (NECA) led to a significant potentiation of Kir3.1F137S currents (control: 35 ± 2 pA pF^{-1}, + 1 μM NECA: 112 ± 4 pA pF^{-1}, wash: 27.5 ± 5 pA pF^{-1}, $n = 6$, $P < 0.001$).

We investigated the effects of the $G_{q/11}$-coupled M_3 receptor upon Kir3.1F137S channels. Similarly, carbachol (10 μM) caused an initial potentiation (control: 50 ± 4 pA pF^{-1}, +carbachol: 142 ± 14 pA pF^{-1}) followed by marked inhibition of currents (25 ± 3 pA pF^{-1}, $n = 12$). Recovery from inhibition was observed within ~8 min (42 ± 4 pA pF^{-1}). This is in contrast to M_3-mediated inhibition of Kir3.1+3.2A channels, which did not exhibit recovery from inhibition (control: 37 ± 9 pA pF^{-1}, +carbachol: 101 ± 15 pA pF^{-1}, wash: 19 ± 5 pA pF^{-1}, 8 min wash: 21 ± 4 pA pF^{-1}, $n = 15$).

Co-expression of $G\beta1\gamma2$ accelerated the recovery of Kir3.1F137S currents from M_3-mediated inhibition such that currents were fully recovered by 5 min. The prolonged inhibition of Kir3.1+3.2A channels was not altered by $G\beta1\gamma2$.

Studies are currently underway to investigate regulation of homomeric Kir3.2 channels.

Chan KW *et al.* (1996). *Proc Natl Acad Sci* **93**, 14193–14198.

Leaney JL *et al.* (2001). *J Physiol* **534**, 367–379.

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PC69

Functional properties of TRPV3: a novel heat-activated member of the vanilloid receptor family

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Mammalian transient receptor potential (TRP) channels include non-selective cation channels such as TRPV1 that are activated by a range of different stimuli including, pH, vanilloids and heat (Caterina *et al.* 1997). It is likely that TRPV1 contributes to the perception of noxious heat. However, the channels involved in thermal sensation between 22 and 40 °C are less well understood and may involve a capsaicin-insensitive receptor (Nagy & Rang, 1999). We have used a combination of bioinformatics and molecular biology to identify novel vanilloid receptor-like channels that may be involved in thermal sensation. TRPV3 was identified as a polypeptide of 790 amino acids with 43 % amino acid identity to TRPV1. This receptor is identical to that recently published by several groups (e.g. Peier *et al.* 2002). Functional properties of this channel were investigated using whole-cell patch-clamp electrophysiology.

Chinese hamster ovary (CHO) cells were transiently transfected with 2 μg ml^{-1} of human TRPV3 cDNA in a pIRES-eGFP vector. Transfected cells were grown on poly-D-lysine-coated glass coverslips and studied using whole-cell voltage-clamp electrophysiology. Temperature changes were generated using a rapid solution heater (MSDRL Research Engineering), over a range of 22–55 °C. All recordings were made at −60 mV in a solution of (mM): 1.67 $CaCl_2$, 1 $MgCl_2$, 2 KCl, 165 NaCl, 17 D-glucose and 10 Hepes; pH 7.3 (NaOH). Patch pipettes (borosillicate glass; 2–6 MΩ) were filled with (mM): 110 CsF, 30 TEA-Cl, 20 Cs-BAPTA, 2 ATP-Mg, 1 $MgCl_2$ and 10 Hepes; pH 7.2 (TEA-OH). Application of pH 5.5 or 10 μM capsaicin did not activate currents in TRPV3-expressing cells (−0.1 ± 2.3 pA, $n = 5$ and 2.5 ± 2.9 pA, $n = 5$, respectively; means ± S.E.M.). In contrast, increasing the temperature of the extracellular solution from 22 to 55 °C (2.8 °C s^{-1}) elicited inward currents (−158.9 ± 40.2 pA; $n = 10$) which were significantly greater than that in cells mock-transfected with empty pIRES-eGFP vector (−24.2 ± 3.9 pA; $n = 8$; $P < 0.01$, Student's unpaired t test). Currents were activated at a threshold of ~38 °C ($n = 10$) and exhibited outward rectification (voltage ramps −80 to +80 mV, 320 mV s^{-1}) with a reversal potential of −6 mV, similar to TRPV1.

These data confirm that TRPV3 is a novel member of the TRPV family that can be activated by heat but not capsaicin or protons. The functional relevance of this channel subtype to thermal sensation and temperature regulation remains to be elucidated.

Caterina MJ *et al.* (1997). *Nature* **389**, 816–824.

Nagy I & Rang HP (1999). *J Neurosci* **19**, 10647–10655.

Peier AM *et al.* (2002). *Science* **296**, 2046–2049.

PC70

Identification of domains mediating $GABA_A$ receptor activation

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Whilst many of the residues that form the agonist binding site and channel lining of $GABA_A$ receptors have been identified, the means by which they transduce agonist binding to channel opening remains to be elucidated. To identify domains that mediate activation of murine $\alpha1\beta1$ $GABA_A$ receptors, chimeric subunits containing sequences from murine and *Drosophila* $GABA_A$ receptor subunits were generated, expressed in HEK 293 cells and studied under whole-cell patch-clamp.

The M2–3 loop of the $\beta1$ subunit was found to play a critical role in receptor activation. Substituting transmembrane domains M1 and M2 in $\beta1$ with the corresponding regions of the *Drosophila* subunit RDL had no effect on agonist potency (mean ± S.E.M. GABA EC_{50} of wild-type $\alpha1\beta1$ receptors = 1.2 ± 0.1 μM, $n = 5$). However, GABA (10 mM, $n = 30$ cells) and pentobarbitone (10 mM $n = 18$ cells) activation was abolished when RDL domains M1–M3 were introduced into $\beta1$ subunits ($\beta1^{\text{RDL M1-M3}}$). This loss of function was dependent upon multiple residues in the N-terminal half of M3. Indirect immunofluorescence assays demonstrated that subunit assembly was unaffected by these substitutions, consistent with the uncoupling of the channel gate from the agonist-binding domain.

Homology modelling of the N-terminal region of $\beta1$ on the structure of the *Lymnaea* acetylcholine binding protein (Brejc *et al.* 2001) identified likely points of contact between the extracellular and transmembrane domains. GABA and pentobarbitone activation of receptors containing the non-functional $\beta1^{\text{RDL M1-M3}}$ subunits were restored by introducing RDL-specific sequences into the disulphide loop that is found in all receptors of this class. The GABA EC_{50} of receptors containing this chimeric subunit (5.1 ± 0.3 μM, $n = 6$) was close to that of wild-type receptors but the increased degree of desensitisation and reduced whole-cell current amplitude exhibited by these receptors (approximately 20 % of wild-type) indicated that other regions of the N-terminal domain also participate in receptor activation. We are currently engaged in identifying these regions.

Our data show a critical interdependence between the primary sequences of the disulphide and M2–M3 loops of the β subunit in $GABA_A$ receptor activation that may reflect a physical interaction between these domains.

Brejc K *et al.* (2001). *Nature* **411**, 268.

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PC71

Co-ordinate patterns of KChIP2 mRNA, protein and I_{to} expression in the canine ventricles

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The transmural gradient in the transient outward current (I_{to}) is an important determinant of the normal sequence of cardiac action potential repolarization. I_{to} is a fast activating and inactivating K^+ current present in the cardiac myocytes of most mammalian species and its density is much higher in the epicardium than in the endocardium of the free ventricular wall.

Recently, we have proposed that the gradient in I_{to} is determined by the uneven expression of the K^+ channel β subunit gene KChIP2 (K^+ channel interacting protein 2) across the ventricular wall. In both human and canine heart, KChIP2 mRNA expression parallels the gradient in I_{to}, while the α subunit Kv4.3 mRNA is expressed evenly across the ventricle (Rosati *et al.* 2001).

A recent study (Deschenes *et al.* 2002) has cast doubt on our hypothesis, reporting that the KChIP2 protein expression is uniform across the canine and human ventricles. We have re-examined this issue and have found that KChIP2 protein levels tightly match ($R^2 = 0.91$) those of KChIP2 mRNA and parallel the density of I_{to} in nine different regions of the canine ventricle.

These conflicting results can be reconciled only by assuming that one set of data is artifactual, i.e. due to the cross-reaction of the anti-KChIP2 antibody with a different protein. Using microarrays, we show that only 0.1% of the genes expressed in human heart are upregulated in the epicardium of the left ventricle relative to endocardium. Therefore, the probability of our results being due to a non-specific cross-reaction is extremely low. This possibility becomes even lower when it is taken into account that the KChIP2 protein distribution matches the KChIP2 mRNA distribution throughout the ventricles.

RNA isolation, RNase protection assays, myocyte preparation and electrophysiological recordings were performed as previously reported (Rosati *et al.* 2001). Western blot experiments were conducted according to Guo *et al.* (2002). The microarray experiment was performed using an Affymetrix Human genome GeneChip Set (U133) and data analysis was conducted according to Irizarry *et al.* (2002). Donor animals were humanely killed. All animal procedures were approved by the Institutional Animal Care and Use Committeee of SUNY at Stony Brook. Human RNA had been previously isolated after approval of the University's ethical committee (Rosati *et al.* 2001).

Deschenes I *et al.* (2002). *Circulation* **106**, r8–14.

Guo W *et al.* (2002). *Circ Res.* **90**, 586–593.

Irizarry RA *et al.* (2002). *Biostatistics* (in the Press).

Rosati B *et al.* (2001). *J Physiol* **533**, 119–125.

All procedures accord with current National guidelines.