

## C1

**Effects of piperine, the pungent ingredient of black pepper, at the human vanilloid receptor (TRPV1)**

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Piperine is a pungent alkaloid found in many species of the genus *Piper*. The structure of this molecule somewhat resembles that of capsaicin, another culinary alkaloid, and the best known of the vanilloid receptor-1 (TRPV1) agonists. Recent studies have demonstrated that piperine can modulate gastrointestinal transit and may even have a gastroprotective effect (Szolcsanyi & Bartho 2001). We describe here our efforts to characterise the effects of piperine at human TRPV1 using electrophysiological methods.

Whole-cell patch-clamp recordings were made from HEK 293 cells stably expressing hTRPV1 (Hayes *et al.* 2000). The extracellular solution consisted of (mM): NaCl, 130; KCl, 5; BaCl<sub>2</sub>, 2; MgCl<sub>2</sub>, 1; glucose, 30; Hepes-NaOH, 25; pH 7.3. Electrodes were filled with (mM): CsCl, 140; MgCl<sub>2</sub>, 4; EGTA, 10; Hepes-CsOH, 10; pH 7.3. Data are expressed as mean  $\pm$  S.E.M., Student's *t* test was used to analyse statistical significance ( $P < 0.05$ ).

Piperine, activated inward currents in HEK 293 cells expressing TRPV1 with an estimated mean  $EC_{50} = 38 \pm 2 \mu\text{M}$  ( $n = 5-9$ ). Neither piperine nor capsaicin had any effect on parental HEK 293 cells. The current activated by piperine ( $30 \mu\text{M}$ ) was reversibly antagonised by the TRPV1 inhibitor capsazepine ( $10 \mu\text{M}$ ),  $94 \pm 5.1\%$  block, ( $n = 3$ ); and the non-competitive TRPV1 blocker ruthenium red ( $10 \mu\text{M}$ ),  $95 \pm 4.7\%$ , ( $n = 4$ ). Over a  $-70$  to  $+70$  mV voltage ramp, the currents elicited by  $30 \mu\text{M}$  piperine and  $1 \mu\text{M}$  capsaicin yielded indistinguishable current-voltage relationships; these were outwardly rectifying with reversal potentials of  $0 \pm 0.4$  mV and  $-1 \pm 0.8$  mV, respectively. A high concentration of piperine ( $100 \mu\text{M}$ ) displayed  $2.86 \pm 0.91$  fold ( $n = 6$ ) greater efficacy than  $1 \mu\text{M}$  capsaicin, a concentration that produces  $\geq 90\%$  maximal response. In addition, different desensitisation profiles were observed with capsaicin  $1 \mu\text{M}$  producing little macroscopic desensitisation during applications lasting 20 s while currents activated by  $100 \mu\text{M}$  piperine desensitised  $50\%$  in  $9.9 \pm 0.7$  s ( $n = 5$ ). Weak acidification modulates TRPV1 activity. At pH 6.5 a similar ( $P > 0.05$ ) degree of potentiation ( $I_{pH6.5}/I_{pH7.3}$ ) was observed for responses to  $1 \mu\text{M}$  capsaicin ( $2.42 \pm 0.32$ ,  $n = 7$ ) and  $30 \mu\text{M}$  piperine ( $2.56 \pm 0.27$ ,  $n = 4$ ).

These data indicate that two different TRPV1-activating alkaloids can produce distinct response phenotypes at hVR1 and suggest that capsaicin may be regarded as a partial agonist of this conductance. Agonist desensitisation strategies may have further applications in treating pain and gastrointestinal disorders.

Hayes P *et al.* (2000). *Pain* **88**, 205–215.Szolcsanyi J & Bartho L (2001). *J Physiol Paris* **95**, 181–188.

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## C2

**Activation and modulation of human vanilloid receptor-1 (hTRPV1) by protons**

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TRPV1 is a non-selective cation channel thought to play a key role in the detection of noxious stimuli by sensory neurons. TRPV1 effectively serves as a molecular integrator of a number of diverse activators including capsaicin, protons and heat ( $>43^\circ\text{C}$ ), however, the physiological (and pathophysiological) activators of TRPV1 *in vivo* are still debated. Protons are known to both directly activate TRPV1 and to cause potentiation of heat or capsaicin-mediated agonism. Consequently, they are likely to be a key endogenous regulator of TRPV1. We have therefore undertaken a detailed investigation of the effects of protons on the hTRPV1 expressed in HEK293 cells using the whole-cell patch clamp technique. Application of protons was achieved by applying a solution of known pH (9.3–4.3) to the cell under study using a rapid perfusion system (see Hayes *et al.* 2000). Data are given as mean  $\pm$  S.E.M. and significance ( $P < 0.05$ ) was ascertained using Student's unpaired *t* test.

Protons behaved as an agonist on hTRPV1 expressing cells leading to the activation of slowly desensitising currents ( $pEC_{50} = 5.21$ ) which were inhibited by the TRPV1 antagonists capsazepine and SB-366791 ( $1 \mu\text{M}$ ). Acid-evoked currents deactivated rapidly upon agonist removal commensurate with a highly cooperative gating mechanism and an externally accessible binding site. Interestingly, a degree of apparent proton-mediated inhibition was also noted at  $pH \leq 5$ . Acidification also caused marked potentiation (from  $3.27 \pm 0.14$ ,  $n = 8$ , to  $13.44 \pm 6.20$  fold,  $n = 7$ , at pH 6.5 and pH 4.3, respectively) of capsaicin-gated currents. This potentiating effect was estimated to be half maximal at  $\sim pH 6.0$ . Equivalent potentiation by pH 6.5 was observed at supramaximal concentrations of capsaicin ( $3.08 \pm 0.03$  fold at  $30 \mu\text{M}$ ) and other agonists such as olvanil ( $100$  nM,  $3.54 \pm 0.11$  fold,  $n = 5$ ) and resiniferatoxin ( $100$  nM,  $3.16 \pm 0.08$  fold,  $n = 5$ ). These data are consistent with a mechanism in which protons mediate a distinct agonist-independent effect on TRPV1 function rather than by altering agonist potency. In further experiments, we noted that the degree of potentiation by protons was related to the rectification properties of hTRPV1 and demonstrated that the acid-mediated potentiation of capsaicin-gated currents was itself strongly voltage-dependent.

In summary, we have shown that proton modulation of the hTRPV1 receptor is complex, with extracellular acidification causing response potentiation, direct gating and a degree of current block as pH is progressively lowered. Our findings imply that the voltage- and time-dependent properties of the receptor and the potentiation of hTRPV1 by protons may be inextricably linked by a common mechanism.

Hayes *et al.* (2000). *Pain* **88**, 207–217.

## C3

### Two biophysically distinct currents activated by $\alpha,\beta$ -methylene ATP in a cell line co-expressing human P2X<sub>2</sub> and P2X<sub>3</sub> channel subunits

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The subunits that make up the P2X receptor family form both homomeric and heteromeric ATP-gated non-selective cation channels. Assemblies of P2X<sub>3</sub> and of P2X<sub>2</sub> and P2X<sub>3</sub> subunits are believed to dominate the P2X receptor complement of sensory neurones, and consequently have been associated with nociceptive signalling and the search for novel analgesics.

We have characterised a CHO cell-line stably expressing both the human P2X<sub>2</sub> and P2X<sub>3</sub> subunits, using whole cell voltage-clamp combined with concentration-jump techniques. Currents mediated by P2X<sub>3</sub> and/or P2X<sub>2</sub>/3 receptors were activated using 10  $\mu$ M  $\alpha,\beta$ -me ATP, a concentration previously demonstrated not to significantly activate homomeric P2X<sub>2</sub> receptors. Data are expressed as mean  $\pm$  S.E.M.

At  $-30$  mV, single 1.5 s applications of 10  $\mu$ M  $\alpha,\beta$ -me ATP, characteristically produced a biphasic current response consisting an early component which activated and desensitised rapidly followed by a sustained plateau current. When a second identical agonist application was made 5 s later the initial desensitising current component was entirely absent, whereas the sustained current component remained largely intact. By subtracting the second response from the first it was possible to characterise the properties of the desensitising current component ( $I_{\text{desen}}$ ), which is likely to reflect P2X<sub>3</sub> homomers, and compare it to the response to the second agonist challenge ( $I_{\text{resis}}$ ), which is likely to represent only P2X<sub>2</sub>/3 heteromers. An agonist free period of around 3–5 min was required to allow  $I_{\text{desen}}$  to completely recover. The ratio of  $I_{\text{desen}}$  to  $I_{\text{resis}}$  varied from 0.34 to 35.8 and did not correlate with current amplitude. The reversal potentials of both  $I_{\text{desen}}$  and  $I_{\text{resis}}$  were indistinguishable from 0 mV.  $I_{\text{resis}}$ , like homomeric P2X<sub>2</sub> currents, exhibited strong inward rectification ( $I_{-60}/I_{+60} = 0.14 \pm 0.02$ ,  $n = 8$ ), whereas,  $I_{\text{desen}}$ , like homomeric P2X<sub>3</sub> channels was only weakly rectifying ( $I_{-60}/I_{+60} = 0.71 \pm 0.08$ ,  $n = 8$ ). At  $-30$  mV, the time constants of  $I_{\text{resis}}$  activation and deactivation did not correlate and were  $565 \pm 33$  ms and  $635 \pm 33$  ms, respectively. Activation was significantly slowed and deactivation significantly speeded by depolarisation, inferring agonist affinity may be reduced by membrane depolarisation. Macroscopic desensitisation of  $I_{\text{resis}}$  occurred with a time constant of  $16.5 \pm 2.8$  s, but failed to reach completion during 90 s of continuous agonist application. Unlike GABA<sub>A</sub> receptors, the deactivation kinetics of  $I_{\text{resis}}$  were not sensitive to prior desensitisation. Subsequent experiments with cultured rat neonatal dorsal root ganglion neurones revealed a similar dual component response to 10  $\mu$ M  $\alpha,\beta$ -me ATP suggesting these cells express both homomeric P2X<sub>3</sub> and heteromeric P2X<sub>2</sub>/3 channels.

## C4

### Molecular determinants of P2X<sub>4</sub> trafficking

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P2X receptors are ionotropic receptors gated by ATP and are composed of two transmembrane domains, an extracellular ligand binding site and intracellular N and C-termini. It has been shown that P2X<sub>4</sub> receptors are rapidly cycled between the cell surface and endosomal compartments and that this internalization is dependent on a non-canonical tyrosine-based endocytic motif contained within the C-terminus of P2X<sub>4</sub> (Royle *et al.* 2002). This endocytic motif mediates the increased internalization that occurs on application of ATP.

CD8 is commonly used as a reporter molecule to study receptor trafficking as it is transported the cell surface and does not internalize. By transplanting the C-terminus of P2X<sub>4</sub> onto CD8 (CD8 4Ct), and following the trafficking of the CD8 4Ct chimera using antibody labelling in live HEK293 cells (from the European Collection of Cell Cultures), we saw that the chimera did internalize. This demonstrates that the C-terminus of P2X<sub>4</sub> contains all the determinants necessary for the internalization of the chimeric protein. Mutation of the endocytic motif within the P2X<sub>4</sub> C-terminus of the chimeric receptor resulted in an increase in surface expression however internalization was not inhibited to the same extent as in the wild-type receptor. Internalization of both P2X<sub>4</sub> and CD8 4Ct was clathrin-dependent and could be inhibited using siRNA directed against clathrin heavy chain. In the P2X<sub>4</sub> receptor C-terminus there are two clusters of lysine residues. Mutation of the first cluster of lysine residues prevented the increased internalization of the wild-type P2X<sub>4</sub> receptor that occurs on ATP application, but trafficking and function appeared to be otherwise unaffected. In contrast, mutation of the lysine residues immediately upstream of the endocytic motif greatly reduced the amount of receptor cycling between the plasma membrane and endocytic compartments. It also made the receptor non-functional, however function does not appear to be a prerequisite for receptor trafficking. The non-functional P2X<sub>4</sub> G347I mutant was constitutively trafficked to and from the plasma membrane similar to the wild-type receptor.

We have used the CD8 4Ct chimera to identify molecular determinants of trafficking. It has the advantage that the effects of mutations can be analysed without concern for changes in receptor conformation or function.

Royle SJ *et al.* (2002). *J Biol Chem* **277**, 35378–35385.

## C5

### Trafficking of heteromeric P2X purinoreceptors

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The P2X receptor subtypes P2X<sub>2</sub>, P2X<sub>4</sub> and P2X<sub>6</sub> have been shown to have an overlapping distribution in the CNS (Rubio & Soto, 2001). Both P2X<sub>2</sub> and P2X<sub>4</sub> can function as homomeric receptors unlike P2X<sub>6</sub> which is non-functional on its own but can form functional heteromers with both P2X<sub>2</sub> and P2X<sub>4</sub>. When expressed individually in mammalian cell lines the P2X receptor subtypes have very different distributions. P2X<sub>2</sub> is stably expressed at the plasma membrane, P2X<sub>4</sub> has a punctate, endosomal-like distribution within the cell and P2X<sub>6</sub> is retained

in the ER. We have used both immunocytochemistry and biotinylation to compare the trafficking of the heteromeric receptors with that of the homomeric.

We introduced extracellular epitopes into all three subtypes to allow detection of surface receptors in HEK293 cells. We found that when P2X<sub>6</sub> was expressed alone most of the receptors were retained within the ER. There was a small amount of surface expression in some cells suggesting that overexpression caused saturation of the retention pathway. When P2X<sub>6</sub> was co-expressed with either P2X<sub>2</sub> or P2X<sub>4</sub> its subcellular distribution changed. Most notably there was a dramatic increase in the number of P2X<sub>6</sub> receptors at the surface. When P2X<sub>6</sub> was expressed with P2X<sub>4</sub>, it was internalised and when it was expressed with P2X<sub>2</sub> it was stable at the cell surface similar to the P2X<sub>2</sub> and P2X<sub>4</sub> homomers.

P2X<sub>6</sub> expressed alone in HEK293 cells gave two products of sizes 40 kDa and 45 kDa. The lower band corresponded to the weight of the unglycosylated receptor and the higher band corresponded to a glycosylated form of the receptor; it disappeared when it was incubated with N-glycosidase. Interestingly, when P2X<sub>6</sub> was expressed with either P2X<sub>2</sub> or P2X<sub>4</sub> an additional product appeared at 46 kDa. This suggests that co-expression with P2X<sub>2</sub> or P2X<sub>4</sub> allowed the carbohydrate groups to be further processed in the Golgi on the way to the cell surface.

Rubio ME & Soto F (2001). *J Neurosci* **21**, 641–653.

## C6

### The N-terminus of the human chloride channel ClC-4 contains an endoplasmic reticulum localisation signal

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The human ClC chloride channel isoforms can be classified in two groups: the plasma membrane isoforms ClC-1, ClC-2, ClC-Ka and ClC-Kb, and the intracellular isoforms ClC-3, ClC-5 and ClC-7. ClC-3 and ClC-5 are localised in endosomes, where they contribute to the anion shunt current facilitating acidification of these compartments by the proton pump (Jentsch, T.J. *et al.* 2002). Little is known about the subcellular localisation of ClC-4. However, since it shares high sequence identity with ClC-3 and ClC-5, we investigated whether ClC-4 is localised in an intracellular compartment.

We transiently transfected ClC-4 conjugated C-terminally with GFP (ClC-4-GFP) in HEK293 cells. Expression of ClC-4-GFP in these cells was predominantly found in a dense intracellular network of membranes identified as the endoplasmic reticulum (ER) by co-staining for the sarco/endoplasmic reticulum ATPase (SERCA).

To identify the motif responsible for ER localisation of ClC-4 we generated chimaeras by exchanging segments of ClC-4 with the corresponding regions of ClC-3, and the unrelated type II plasma membrane protein Ly49E. Replacement of the C-terminal tail of ClC-3 with the corresponding sequence of ClC-4 did not change its vesicular localisation, suggesting that the putative ER retrieval motifs in the cytoplasmic C-terminus of ClC-4 are not responsible for its ER localisation. In contrast, a chimaera in which the first 71 N-terminal amino acids of ClC-3 were replaced with the corresponding segment of ClC-4 showed a localisation similar to ClC-4, as confirmed by co-localisation with SERCA. To exclude that the ER localisation of this chimaera was the consequence of ER retention due to misfolding, instead of being due to a genuine ER localisation signal, we created a mirror-

image chimaera where the first 71 N-terminal amino acids of ClC-4 were substituted with the equivalent segment of ClC-3. Indeed, this chimaera showed a vesicular distribution pattern, suggesting that the N-terminus of ClC-4 contains a genuine ER localisation signal. This was confirmed by the observation that substitution of the cytoplasmic N-terminus of the plasma membrane protein Ly49E with the first N-terminal 63 amino acids of ClC-4 redistributed a large amount of the expressed protein from the plasma membrane to the ER. Moreover, no alteration of the ER localisation was found, when ClC-4 was truncated at amino acid 112 after the first transmembrane spanning domain.

Taken together, our results indicate that the first 63 N-terminal amino acids of ClC-4 contain a novel ER targeting signal that is both necessary and sufficient for localisation in the ER.

Jentsch TJ *et al.* (2002). *Physiol Rev* **82**, 503–568.

## C7

### Mutations of the selectivity filter of the tandem pore K<sup>+</sup> channel TASK-1 that affect ionic selectivity

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A triplet of residues, GYG, forms part of the consensus sequence of the pore domain and generates the selectivity of potassium channels. However, the two pore domains of tandem pore K<sup>+</sup> channels lack sequence identity: P1 has GYG in all cases, (except TREK-1); P2 shows greater variation and has GFG in TASK-1. Do these two regions have similar effects on ionic selectivity? We have made substitutions in the triplet of residues in P1 and P2 of murine TASK-1. Channels were expressed in oocytes taken from *Xenopus* frogs that had been anaesthetised by immersion in 0.3% w/v MS222 and killed humanely by destruction of the brain and spinal cord. Two-electrode voltage clamp was used to measure the shift in reversal potential when K<sup>+</sup> in the external medium was replaced by Rb<sup>+</sup> or Na<sup>+</sup>.

We made the mutations Y96 → F, L, V, and M in P1, but only Y96F generated functional channels. Surprisingly, this mutation exhibited significantly altered selectivity compared to wild-type.  $P_{Rb}/P_K$  was increased from  $0.77 \pm 0.02$  ( $n = 6$ ) to  $0.99 \pm 0.02$  ( $n = 10$ ; mean  $\pm$  S.E.M.;  $P < 0.001$ , using ANOVA). Unlike wild-type, the channel was noticeably Na<sup>+</sup>-permeant, with an increase in  $P_{Na}/P_K$  from  $0.02 \pm 0.003$  ( $n = 6$ ) to  $0.44 \pm 0.03$  ( $n = 10$ ;  $P < 0.001$ ). The equivalent residue in P2, F202, was more tolerant of substitution since the mutations F202 → L, V, M, Y, and A all generated functional channels. Selectivity was reduced in every case; e.g. F202M gave  $P_{Rb}/P_K = 0.92 \pm 0.03$  and  $P_{Na}/P_K = 0.65 \pm 0.05$  ( $n = 10$ ). The permeability ratio for Rb<sup>+</sup>/K<sup>+</sup> was significantly increased over wild-type ( $P < 0.01$ ).

The Gly residues of the selectivity sequence are thought to be particularly important for selectivity (Heginbotham *et al.* 1994). We replaced each Gly in P1 and P2 in turn by Ala. Such mutant channels were non selective. In P1, G97A gave channels with  $P_{Rb}/P_K = 0.89 \pm 0.05$  and  $P_{Na}/P_K = 0.71 \pm 0.09$  ( $n = 8$ ). The equivalent mutation in P2, G203A, gave  $P_{Rb}/P_K = 0.95 \pm 0.05$  and  $P_{Na}/P_K = 0.78 \pm 0.12$  ( $n = 7$ ). Similar alterations in selectivity were seen with G95A and G201A.

Our results are similar to those obtained on mutation of the equivalent residues of voltage gated K<sup>+</sup> channels (Heginbotham *et al.* 1994), though the effects of substitution of Y by F (or vice versa in P2) are greater. In contrast in inward rectifier K<sup>+</sup> channels, mutations of the residues of the GYG triplet in two pore forming regions (out of the four in each channel) results in

channels with unaltered selectivity (Dart *et al.* 1998; So *et al.* 2001).

Dart C *et al.* (1998). *J Physiol* **511**, 25–32.

Heginbotham L *et al.* (1994). *Biophys J* **66**, 1061–7.

So I *et al.* (2001). *J Physiol* **531**, 37–50.

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All procedures accord with current UK legislation

## PC2

### A human protein with similarity to the *Drosophila* dSLIP1 protein regulates trafficking of BK<sub>Ca</sub> channels

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In *Drosophila* and higher animals the large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK<sub>Ca</sub>) channel is involved in regulating the membrane potential of both excitable and non-excitable cells. In mammalian central neurons, BK<sub>Ca</sub> channels co-localise with N-type calcium channels (Marrion & Tavalin, 1998), and are mainly targeted to axons and nerve terminals (Knaus *et al.* 1996). The mechanisms by which this potassium channel is targeted to discrete sub-cellular regions are not understood. We have identified a human cDNA derived from human brain that encodes a protein similar to *Drosophila* dSLIP1 (Xia *et al.* 1998), a protein that interacts with the dSlo BK<sub>Ca</sub> channel. The human isoform, hSLIP1, is a cytoplasmic protein of 1098 amino acids, possesses two PDZ domains, and interacts directly with the human BK<sub>Ca</sub> channel subunit hSloα1, which we determined by the co-immunoprecipitation of subunits co-expressed in HEK cells. Like its *Drosophila* counterpart hSLIP1 significantly ( $P < 0.01$ ,  $t$  test) reduced macroscopic BK<sub>Ca</sub> currents when co-expressed with hSloα1 in *Xenopus* oocytes. The current evoked by a depolarising pulse to +120 mV was (mean ± S.E.M.)  $9.03 \pm 0.86 \mu\text{A}$  ( $n = 7$ ) in oocytes expressing hSloα1 alone and  $4.37 \pm 0.56 \mu\text{A}$  ( $n = 6$ ) in oocytes co-expressing hSloα1 and hSLIP1. This property of hSLIP1 was prevented by co-injecting mRNA encoding the fragment F<sub>C</sub>, the last 142 amino acids of the hSloα1 intracellular C-terminus ( $9.99 \pm 1.56 \mu\text{A}$ ,  $n = 5$ ), implicating this domain in interacting with hSLIP1. Co-expressing hSLIP1 or F<sub>C</sub> with hSloα1 in oocytes had no significant effect on channel gating or unitary conductance: in inside-out patches the activation  $V_{1/2}$  was  $10.0 \pm 5.2$  mV,  $20.2 \pm 5.4$  mV, and  $17.5 \pm 2.6$  mV in  $10 \mu\text{M}$   $[\text{Ca}^{2+}]_i$  ( $n = 5$ ) and conductance was  $279 \pm 5$  pS,  $265 \pm 7$  pS, and  $264 \pm 5$  pS ( $n = 3$ ) in symmetrical 140 mM K<sup>+</sup> for hSloα1, hSloα1 + hSLIP1, and hSloα1 + F<sub>C</sub>, respectively. We therefore hypothesise that hSLIP1 is involved in regulating the surface expression and sub-cellular distribution of BK<sub>Ca</sub> channels.

Knaus HG *et al.* (1996). *J Neurosci* **16**, 955–63.

Marrion NV & Tavalin SJ (1998). *Nature* **396**, 900–5.

Xia X *et al.* (1998). *J Neurosci* **18**, 2360–9.

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All procedures accord with current UK legislation

## PC3

### The effects of molecular domains I to IV on the activation of the T type calcium channel Ca<sub>v</sub>3.1

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The main subunits of voltage-dependent calcium channels have four domains (I–IV), each comprising six transmembrane regions (S1–S6). Calcium channels are classified into low-voltage activated (LVA) and high-voltage activated (HVA) channels. Ca<sub>v</sub>3 (T type) channels are low-voltage activated, while Ca<sub>v</sub>1 (L type) and Ca<sub>v</sub>2 (N, P/Q and R type) channels are high-voltage activated. The aim of this study is to investigate the molecular basis for the difference in voltage-dependence of activation between LVA and HVA channels.

For this, cDNA chimeras were made between a LVA channel, Ca<sub>v</sub>3.1 (α<sub>1G</sub>), and a HVA channel, Ca<sub>v</sub>1.2 (α<sub>1C</sub>), by using overlap extension PCR and standard molecular biological methods. Referring to the four domains of Ca<sub>v</sub>1.2 wild-type as CCCC and the wild-type Ca<sub>v</sub>3.1 as GGGG, four chimeras (CGGG, GCGG, GGCG and GGCG) were constructed. cRNAs for these constructs were injected into *Xenopus* oocytes, together with α<sub>2</sub>/δ and β subunits. Two-electrode voltage-clamp recordings were made 2–3 days after injection. To obtain current voltage (I/V) curves, depolarising pulses were applied (0.1 Hz, 500 ms duration) from a holding potential of –80 mV, with Ba<sup>2+</sup> as charge carrier. The voltage for half maximal activation ( $V_{0.5}$ ) and slope parameter ( $k$ ) were obtained from Boltzmann fits.

For wild-type channels, as expected, Ca<sub>v</sub>3.1 activated at more negative potentials ( $V_{0.5} -40.8 \pm 2.5$  mV,  $n = 11$ , mean ± S.E.M.) than for the high-voltage activated Ca<sub>v</sub>1.2 ( $V_{0.5} 6.9 \pm 0.9$  mV,  $n = 10$ ). The three chimeras CGGG, GGCG and GGCG activated at high voltages with I/V curves like Ca<sub>v</sub>1.2, and indeed the voltages for half-maximal activation for these chimeras were not significantly different from wild-type Ca<sub>v</sub>1.2 ( $V_{0.5} 5.2 \pm 0.5$  mV,  $n = 8$ , CGGG;  $4.6 \pm 0.9$  mV,  $n = 9$ , GGCG; and  $5.8 \pm 1.0$  mV,  $n = 7$ , GGCG, Student's  $t$  test). Furthermore, the slope parameters  $k$  for these chimeras were also not significantly different from wild Ca<sub>v</sub>1.2. However, chimera GCGG activated at low voltages with I/V curves like Ca<sub>v</sub>3.1, with a value for  $V_{0.5} -40.0 \pm 1.3$  mV,  $n = 9$  that was not significantly different from that for Ca<sub>v</sub>3.1. The  $k$  value for GCGG was also not significantly different from that for Ca<sub>v</sub>3.1.

According to current ideas of channel gating, following depolarisation, S4 segments from all four domains must move before the channel can be opened. Our data for three of our chimeras are consistent with this: replacing domains I, III and IV of a LVA channel with the corresponding domain of a HVA channel led to a HVA channel. However, swapping domain II did not alter the voltage dependence of activation, indicating that domain II somehow plays a different role in channel gating from the other calcium channel domains.

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All procedures accord with current UK legislation

## PC4

**Characterisation of an ADP-Ribose-induced current in cultured rat striatal neurones**

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NAD<sup>+</sup> and H<sub>2</sub>O<sub>2</sub> activate a non-selective cation channel in striatal neurones (Smith *et al.* 2003). This conductance exhibits many similarities to the recently cloned TRPM2 channel and the NSNAD channel expressed by rat CRI-G1 insulinoma cells (Herson *et al.* 1999; Perraud *et al.* 2001). Here, we examine whether ADP-ribose, an activator of TRPM2 and NSNAD, induces a similar current in striatal neurones.

Cultured striatal neurones were prepared from humanely sacrificed E18 rat embryos. Patch clamp recordings were made at ~23°C after 7–14 days *in vitro*. Cells were usually voltage-clamped at –70 mV in either the whole-cell or outside-out patch configurations. The recording pipette contained (mM) kgluconate 140, NaCl 10, Na<sub>2</sub>ATP 4, Na<sub>2</sub>GTP 0.1, Hepes, 10, pH 7.2. As required this was supplemented with 0.5 mM ADP-ribose. The bath solution contained (mM) NaCl 140, KCl 5, MgCl<sub>2</sub> 2, CaCl<sub>2</sub> 1, Hepes 10, pH 7.4. CaCl<sub>2</sub> was replaced with 1 mM BaCl<sub>2</sub> when required. Data are expressed as mean ± S.E.M.

Whole-cell recordings with ADP-ribose in the pipette exhibited a time-dependent development of inward current ( $I_{\text{max}} = -340 \pm 60$  pA,  $n = 23$ ). In all cells replacement of extracellular Ca<sup>2+</sup> with Ba<sup>2+</sup> lead to a substantial decrease in inward current; a similar reduction was also produced by buffering extracellular Ca<sup>2+</sup> with 5 mM EGTA. In control experiments, without ADP-ribose in the recording pipette, a comparable Ba<sup>2+</sup>-sensitive current consistently failed to arise ( $n = 18$ ). The current-voltage relationship of the ADP-ribose activated current was linear, with a reversal potential of  $-6 \pm 3$  mV ( $n = 8$ ). This is indicative of a non-selective cation conductance, like that of TRPM2 and most other TRP channels. This single channel currents underlying the ADP-ribose response could be seen in both whole cell recordings and excised patches. These exhibited openings that often lasted seconds. The mean single channel slope conductance was  $90 \pm 5$  pS ( $n = 10$ ). Channel activity could be reversibly blocked by extracellular application of 0.5 mM LaCl<sub>3</sub> ( $n = 4$ ). In both whole-cell and excised patch recordings, the ADP-ribose-activated current slowly declined in amplitude. This decay developed with a mean time constant of about 400 seconds ( $n = 6$ ) and was incomplete, plateauing at around 55 % of the peak response.

The current described here shares many properties with those evoked by H<sub>2</sub>O<sub>2</sub> and NAD<sup>+</sup> in striatal neurones. Moreover these properties match those of heterologously expressed recombinant TRPM2. These parallels suggest TRPM2 channels may be functionally expressed in the CNS neurones.

Herson PS *et al.* (1999). *JBiolChem* **274**, 833–841.

Perraud AL *et al.* (2001). *Nature* **411**, 595–599.

Smith MA *et al.* (2003). *J Physiol* **547**, 417–425.

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*All procedures accord with current UK legislation*

## PC5

**Extended N-terminal sequence of mTRPC2 and the ubiquitous expression of non-channel transcripts in mouse and human tissues**

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The mTRPC2 channel is essential for function of the mouse vomeronasal organ (VNO) and blockade of this channel in murine sperm results in a reduction in Ca<sup>2+</sup> influx and concomitant reduction in the rate of acrosome reaction. But due to the diverse array of mRNA species derived from the mTrpc2 locus there is controversy surrounding the sites of mRNA expression the nature of the open reading frame of these transcript and hence sites of channel translation and function. In order to address these questions we have undertaken a detailed analysis of the murine trpc2 locus.

We initially identified two further 5' coding exons in the murine *trpc2* locus that have allowed us to extend the coding region of the *trpc2* transcripts derived from the distal promoter in the locus. In addition to extending the open reading frame of the channel encoding mRNA species derived from this promoter these additional exons introduce an upstream open reading frame that would be available for translation in Trpc2 transcripts that are expressed in multiple tissues. Unlike the mRNAs that encode channel proteins these mRNA species include intronic sequence that would introduce stop codons so preventing the translation of a channel protein from these transcripts. A novel feature of this new reading frame is that it is conserved and expressed in the human, while the Trpc2 channel transcripts are not. So while the human lacks Trpc2 transcripts that can encode a channel, this non-channel transcript encodes a protein that is highly conserved between mouse and human. A comparison of the human-mouse synteny maps indicates that a break occurs within the *trpc2* locus and it may be this event, rather than neutral selection, that underlies the loss of the *trpc2* channel transcripts in the higher primates.

*All procedures accord with current national and local guidelines*