C5

TRPC1 is expressed in the plasma membrane of human platelets and associates with TRPC 4 and 5 but not 3 or 6

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We have previously reported that the human homologue of the *Drosophila* transient receptor potential protein hTRPC1 is involved in conducting store-operated Ca^{2+} entry (SOCE) in human platelets. An antibody raised against the pore-forming region of hTRPC1 inhibited Ca^{2+} entry evoked by Ca^{2+} store depletion using thapsigargin and that evoked by the physiological agonist thrombin (Rosado et al. 2002). Furthermore, coimmunoprecipitation experiments show that hTRPC1 associates *de novo* with the type II inositol 1,4,5-trisphosphate receptor when the intracellular Ca^{2+} stores are depleted. We have suggested that this coupling event might underlie the activation of SOCE in platelets. However, others have reported that hTRPC1 is localised in internal membranes in platelets, questioning its role in SOCE (Hassock et al. 2002). Here we have investigated plasma membrane expression of hTRPC1 in human platelets and, using a range of specific antibodies, have probed for the presence of other TRPC proteins in these cells and have investigated their associations.

Biotinylation revealed the presence of hTRPC1 in the plasma membrane of resting platelets. Surface expression was not detectibly changed following depletion of the intracellular Ca^{2+} stores using 1 μM thapsigargin together with 50 nM ionomycin or when the cells were stimulated with 1 unit/ml thrombin (n = 4).

In addition to hTRPC1, Western blotting demonstrated the presence of hTRPC3, hTRPC4, hTRPC5 and hTRPC6 in platelet lysates (n = 6). hTRPC4 and hTRPC5 coimmunoprecipitated with hTRPC1 whereas hTRPC3 and hTRPC6 did not. However, hTRPC3 did coimmunoprecipitate with hTRPC6 and *vice versa*. hTRPC4 and hTRPC5 were found to be associated with detergent-resistant platelet membranes, from which they were partially released when the cells were depleted of cholesterol by treatment with methyl-β-cyclodextrin (MBCD) (n = 5), as we have previously shown for hTRPC1 (Brownlow et al. 2004). In contrast, the distributions of hTRPC3 and hTRPC6 between soluble and membrane fractions were not affected by MBCD treatment.

These results suggest that hTRPC1, hTRPC4 and hTRPC5 form a heteromultimer that is associated with platelet lipid raft domains. This might mediate SOCE. hTRPC6, which has been suggested to conduct store-independent (non-capacitative) Ca^{2+} entry in platelets, may form another heteromultimer with hTRPC3.

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Supported by the Wellcome Trust (064070).

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

C6

Time-dependent changes of the TASK-3 channel expression pattern of melanoma cells maintained in tissue culture: is there a connection between cell-division and TASK-3 expression?

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TASK-3 channels belong to the twin-pore-domain acid-sensitive K^+ channel family and show close relationship with the TASK-1 and TASK-5 channels. It has been proposed that TASK-3 channels may have roles in the genesis and/or development of certain malignant tumours, although their exact roles are still not understood. In this study immunocytochemistry was performed to investigate the TASK-3 expression pattern of three melanoma cell lines maintained in tissue culture. The immortalised melanoma cell lines employed in the present study were obtained from either a primary melanoma tumour (WM35) or from its metastases (HT168 and M1) (for more details see Timar et al. 1999).

The identity of the cells was demonstrated by using melanoma-specific markers (HMB45 and S100 protein). The validity of the present findings was confirmed by employing three different primary antibodies targeting different epitopes of the human TASK-3 channel and by applying preadsorption control experiments. All the findings described in this work were consistently reproducible with all three primary antibodies.

All three melanoma cell lines demonstrated intense TASK-3-specific labelling, whose pattern and distribution showed time-dependent changes. The TASK-3 specific reaction was never confined to the surface membrane of the cells, but intense labelling could be demonstrated intracellularly as well. It was noted that the dividing, spherical cells exhibited an intense, homogenously distributed TASK-3-specific labelling in all three cell lines. The flat, multiprocessal cells, on the other hand, showed somewhat less powerful labelling, which mainly concentrated to the nuclear-perinuclear region of the cell as well as to the developing processes. Rather surprisingly, intense TASK-3-specific labelling could also be demonstrated within the nuclei of the melanoma cells in all three cell lines. The nuclear expression was the most prominent in the proliferating areas (in 10, low cell density visual fields the ratio of the TASK-3 positive nuclei was 99%; n = 280), whereas in the confluent regions only 14% of the nuclei showed TASK-3 positivity (n = 1,216).

When the cell lines were exposed to an antimytotic agent (Mitomycin C) for 48 h, the proportion of the TASK-3-positive nuclei was markedly reduced, especially in the non-confluent areas. When 10-10 low density visual fields were compared, the proportion of the TASK-3 positive nuclei was 30 and 96% in the Mitomycin C-treated and control cultures, respectively. The effect of Ruthenium Red (RR) was also tested, as this substance is an inhibitor of the TASK-3 channels. Besides reducing the number of the surviving melanoma cells in a dose-dependent fashion, RR application induced prominent changes in the cell morphology: the proportion of the multipolar cells was reduced and 98% of the cells showed a distinct spherical appearance (100 mμM). Our results suggest that the inhibition of the cell-division affects the expression pattern of the TASK-3 channels, whereas interfering with the function of the TASK-3 channels may

alter the cell development. These findings imply that there might be a connection between the TASK-3 channels and the cell-division.

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

C7

Ahnak is critical for cardiac Ca(v)1.2 calcium channel function and its beta adrenergic regulation

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Defective L-type Ca^{2+} channel (I_{CaL}) regulation is one major cause for contractile dysfunction in the heart. The I_{CaL} is enhanced by sympathetic nervous stimulation: via activation of beta-adrenergic receptors, PKA phosphorylates the α1C(Ca_V1.2)- and β2-channel subunits and ahnak, an associated 5643-amino acid (aa) protein (Haase et al. 1999). Herein, we examined the role of a naturally occurring, genetic variant I5236T-ahnak on I_{CaL} elicited under whole-cell patch-clamp conditions on ventricular myocytes obtained from humanely killed 3 month old Sprague Dawley rats. On the native cardiomyocytes, intracellular application of 10 μM of the synthetic ahnak peptide carrying the I5236T mutation (GGLPGTGVQGLE) increased I_{CaL} amplitude from 11.9±0.8 to 18.9±1.4 pA/pF ($P<0.05$; n=18,20) at 0 mV depolarization, slowed the inactivation together with a leftward shift in the current-voltage relationship. Importantly, the mutated I5236T-peptide prevented specifically the further upregulation of I_{CaL} by the beta-adrenoceptor agonist, isoprenaline. Under the same experimental conditions, the respective wild-type peptide did not mimic the isoprenaline effects on I_{CaL}. Equilibrium binding experiments performed with recombinant β2 subunit and the ahnak-C1 fragment (aa 4646-5288) by analytical ultracentrifugation revealed a dissociation constant for the ahnak-C1/β2 subunit complex of 155±31 nM (n=14). The binding affinity between ahnak-C1 and β2 subunit decreased by ~50% following PKA phosphorylation of both protein partners. A similar decrease in ahnak-C1/β2 subunit binding affinity was induced by the presence of 10 μM mutated peptide (but not wild-type peptide) in equilibrium binding assays. Hence, we suggest the ahnak-C1 domain serves as a physiological brake on I_{CaL}. Relief from this inhibition is proposed as a common pathway used by sympathetic signalling and I5236T-ahnak fragments to increase I_{CaL}. This genetic ahnak variant might cause individual differences in I_{CaL} regulation upon physiological challenges or therapeutic interventions.

Haase et al. (1999). FASEB J 13, 2161-2172.

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

C8

Quantitative relation between membrane PIP_2 and muscarinic inhibition of M-current in rat sympathetic neurons

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KCNQ(Kv7)/M potassium channels are 'gated' by membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) (Zhang et al. 2003). Hydrolysis and depletion of PIP_2 probably explains the inhibition of these channels produced by stimulating muscarinic acetylcholine receptors (mAChRs) (Suh et al. 2004). We previously reported that, in rat sympathetic neurons, over-expression of the PIP_2-synthesizing enzyme PI5-kinase reduced inhibition of M-channels by the mAChR-agonist oxotremorine-M (Oxo-M) (Winks et al. 2003). We have now assessed quantitatively how far this effect was due to increased membrane PIP_2 levels.

Sympathetic neurons were dissociated from rat superior cervical ganglia (isolated from rats killed according to Home Office regulations), cultured in vitro, and transformed to express the PIP_2-binding fluorophore GFP-PLCδ-PH (Stauffer et al. 1998). Since this construct also binds IP_3 (e.g. Hirose et al. 1999) we used an 'IP_3-displacement' assay to estimate membrane [PIP_2]. For this, we patched neurons with pipettes containing different concentrations of IP_3 and measured the fractional translocation of GFP-PLCδ-PH from the membrane to the cytosol. Using the binding constants of Hirose et al. (1999), mean resting [PIP_2] (95% confidence limits in brackets) as 'seen' by the probe was calculated to be 261 (192-381) μM under control conditions and 693 (457-1153) μM after over-expressing PI5-kinase (Winks et al. 2003).

Oxo-M also induces translocation of GFP-PLCδ-PH (Winks et al. 2003). From this, we calculated the changes in membrane [PIP_2] accompanying mAChR stimulation by incorporating measurements of the effective cytosolic volume available to IP_3 (determined from cell size and comparison of intracellular and aqueous vesicle BODIPY fluorescence) and the membrane:cytosol volume ratio. Expected changes in M-current amplitude were then calculated using the data of Zhang et al. (2003) for KCNQ2/3 channel activation by DiC8-PIP_2 (EC_{50} 87 μM). The calculations accorded with the experimentally-observed concentration-M-current inhibition curve for Oxo-M (mean IC_{50} 1.6 μM; maximal inhibition 74%; ~83% fall in membrane [PIP_2]). They also predicted a much smaller fall in [PIP_2] (23%) in PI5-kinase over-expressing neurons, and much less maximal M-current inhibition (2.3%), in accord with previous measurements (4.6%: Winks et al. 2003). This provides quantitative support for the hypothesis that, in these neurons, mAChR-induced M-current inhibition results directly from membrane PIP_2 depletion.

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Supported by the UK MRC and the Wellcome Trust.

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

SA5

Structure and Dynamics of Voltage-Sensing Ion Channels in the Lipid Bilayer

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The fundamental processes that underlie ion channel function are permeation/selectivity and gating. In an effort to understand ion channel gating, we have used an approach that combines reporter-group spectroscopic techniques (spin labeling/EPR) and electrophysiological methods with classical biochemical and molecular biological procedures. Through site-directed spin labeling, cysteine chemistry was used to introduce nitroxide radicals into specific sites within these channels with high reactivity and specificity. EPR spectroscopy analysis of the spin labeled mutants yields two types of structural information: 1) mobility and solvent accessibility of the attached nitroxide through collisional relaxation methods and 2) distances between pairs of nitroxides through dipole-dipole interactions.

The crystal structures of the bacterial voltage-dependent K+ channel (KvAP) and its isolated voltage-sensor domain have raised several interesting questions about the relative orientation of the membrane domains and the loop regions within the voltage-sensor. Here, we will focus on the structure and dynamics of the voltage-dependent K channel KvAP in the context of the lipid bilayer. We show in reconstituted full-length KvAP that the S4 segment lies at the protein-lipid interface, with most of the gating charges protected from the lipid environment. Additionally, the segment is highly flexible, consisting of two helices separated by a short linker. Accessibility and dynamics data position the S1 segment at the contact interface between the voltage sensing and pore domains.

Analysis of our EPR measurements of the isolated voltage-sensor domain correlates well with the helical nature of several TM segments as predicted by its crystal structure. The probe dynamics and accessibility data show that the S4 segment is highly flexible and is exposed to lipid environment, as in the full-length channel. However, in sharp contrast to the full-length channel, the S1 segment shows significantly higher mobility and oxygen accessibility, which appears to be due to lack of the pore domain which is in close proximity to S1. Our results confirm that S1 in the full-length channel is protected by a protein environment while S4 is exposed to lipids.

These results establish the general principles of voltage-dependent channel structure under physiological conditions and thus limit the types of structural models underlying voltage-dependent gating.

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Cuello, L. G., Cortes, D. M., and Perozo, E. (2004) Molecular architecture of the KvAP voltage-dependent K+ channel in a lipid bilayer Science 306:491-495.

This work is funded by the National Institutes of Health

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

SA6

Lipids and Kv channels - keep the pore open or shut it down

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Voltage-gated potassium (Kv) channels control action potential repolarization, interspike membrane potential, and action potential frequency in excitable cells. It is thought that the combinatorial association between distinct a and b subunits determines whether Kv channels function as non-inactivating delayed rectifiers or as rapidly inactivating A-type channels. It is show that membrane lipids can convert A-type channels into delayed rectifiers, and vice versa. Phosphoinositides such as PIP2 remove N-type inactivation from A-type channels by immobilizing the inactivation domains. Conversely, arachidonic acid and its amide anandamide endow delayed rectifiers with rapid voltage-dependent inactivation by inducing collapse of the selectivity filter of the open pore. The bidirectional control of Kv channel gating by lipids is thought to provide a mechanism for the dynamic regulation of electrical signaling in the nervous system.

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

SA7

Phospholipid-Kir channel interaction in a defined system

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All eukaryotic Kir channels are activated by addition of phosphatidylinositol-4,5-bisphosphate (PIP2) or other negative phospholipids to the cytoplasmic face of the membrane, and particular channel residues have been ascribed to involvement in this activation. However, rigorous determination of the quantitative and physical nature of the channel-PIP2 interaction has been elusive given the unavailability of pure recombinant Kir protein in a reconstituted lipid membrane.

We have used an 86Rb flux assay to characterize the activity of a purified bacterial Kir channel reconstituted into liposomes (Enkvetchakul et al., 2004. J. Biol. Chem., 279, 47076-47080). The additional availability of a crystal structure for this KirBac1.1 channel (Kuo el al., 2003. Science. 300:1922-1926), now paves the way for a rigorous molecular analysis of channel PIP2 interaction.

Strikingly, and in contrast to eukaryotic Kir channels, KirBac1.1 is inhibited by PIP2 and phosphatidylinositol-4-phosphate (PIP) when incorporated at low levels into liposomes. At 0.1% of total lipid (m/m), PIP2 inhibits 86Rb uptake by ~60%, PIP inhibits uptake by ~30%, and there is no significant inhibition by phosphatidylinositol (PI). By contrast, PIP2 is without effect on KcsA-driven Rb-uptake, at up to 3% of total lipid. At up to 1% total

lipid, DAG was without effect on KirBac1.1, and there is no effect of exogenous (100 mM) inositol-1,4,5-triphosphate (IP3). The lipid tail and head-group requirement, as well as the phosphorylation-state dependence of inhibition indicate that, as argued for eukaryotic Kir channels, KirBac1.1 interactions with PIP2 are steric and electrostatic in nature. Multiple positive charges in the cytoplasmic domain of KirBac1.1 are involved in regulation of channel activity, and ongoing mutagenesis of potential phospholipid-interacting residues will now clarify the structural details of PIP2-Kir interaction.

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

SA8

The mechano-gated K2P channels and their regulation by cellular lipids

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The K2P channels are highly conserved from C. elegans to humans with 15 genes identified. They are structurally distinct from other K channel family members, with four transmembrane segments and 2P domains in tandem. K2P channels are homo- or hetero-dimers . The 15 human K2P channels are classified into 6 distinct structural and functional subfamilies named TWIK-, THIK-, TREK-, TASK-, TALK- and TRESK-. K2P channels are expected to play a dominant role in cell electrogenesis, controlling the resting membrane potential, as well as the action potential duration (1).

The TREK subfamily includes TREK-1, TREK-2 and TRAAK. TREK-1 is predominantly expressed in the central and peripheral nervous system, with a particularly strong expression during early development TREK-1 is activated by membrane stretch and cell swelling (2). In the inside-out patch configuration, negative pressure is significantly more effective than positive pressure, suggesting that a specific membrane deformation (convex curving) preferentially opens TREK-1 (2). Mechanical force is transmitted directly to the channel via the lipid bilayer. Intracellular acidosis strongly sensitizes TREK-1 to membrane stretch, leading to channel opening at atmospheric pressure (3).

TREK-1 is reversibly opened by polyunsaturated fatty acids (PUFA), including arachidonic acid (AA) (2). Activation of TREK-1 by AA in the excised patch configuration indicates that the effect is direct by interacting either with the channel protein or by partitioning into the lipid bilayer. Anionic amphipaths, including AA, insert in the outer leaflet of the bilayer and cause a convex curving of the membrane that opens TREK-1. On the contrary, the cationic amphipaths, including chlorpromazine (CPZ) and the local anesthetic tetracaine, insert in the negatively charged inner leaflet and reverse TREK-1 activation (2). Lysophospholipids (LP) including lysophosphatidylcholine (LPC), open TREK-1. The conic shape, rather than the charge of the molecule, is important for lysophospholipid activation. Other membrane phospholipids including PIP2, are also potent openers of TREK-1 (4).

The recent invalidation of TREK-1 in a mouse model demonstrates that it is important for neuroprotection against epilepsy and ischemia. Futhermore, TREK-1 -/- mice are also more resistant to volatile general anesthetics, indicating a role for TREK-1 in the mechanism of general anesthesia (5).

Mutagenesis studies have determined that the cytosolic carboxy terminal domain of TREK-1 plays a key role in TREK-1 gating by both physical and chemical stimuli (2,3). Protonation of a key residue in this region, E306, leads to channel activation (3). Interaction of the carboxy terminal domain of TREK-1 with phospholipids in the inner leaflet of the lipid bilayer is enhanced by protonation of E306 and sensitizes TREK-1 to membrane stretch (3, 4). Conversely, down-modulation of TREK-1 is achieved by receptor- coupled protein kinase A (PKA) phosphorylation of residue S333 (2).

In conclusion, the TREK and TRAAK channels are polymodal K channels that integrate multiple physical and chemical stimuli. Cellular lipids are key regulators of these channels that may play an important functional role in neuroprotection.

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We are grateful to the CNRS, La ligue contre le cancer, l'ARC and The Foundation Paul Hamel for continued support.

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

SA9

Lipid microdomains and the compartmentation of arterial K_{ATP} channel signalling

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In the vasculature, ATP-sensitive K^+ (K_{ATP}) channels are critical regulators of arterial tone, forming a focal point for signalling by many vasoactive transmitters that alter smooth muscle contractility and so vascular resistance and blood flow. Opening of arterial K_{ATP} channels causes membrane hyperpolarization, a decrease in Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels, and vasorelaxation. Thus, vasodilators open K_{ATP} channels, while vasoconstrictors close them and, clinically, these channels form useful targets for drugs designed to treat angina pectoris and hypertension. However, while the biochemical basis of K_{ATP} channel modulation is well-studied, little is known about the structural or spatial organisation of the numerous signalling pathways that converge upon these channels. Here we will discuss evidence that arterial smooth muscle cells utilize specialized lipid microdomains as a means of segregating and organising the complex regulatory pathways that modulate K_{ATP} channel activity.
Many endogenous vasodilators act at receptors coupled to the G protein Gs to elevate K_{ATP} channel activity via activation of cyclic AMP-dependent protein kinase (PKA). Indeed, even in the absence of vasodilators, PKA exerts a steady-state activation of K_{ATP} channels that arises from sustained cAMP production originating from basal adenylyl cyclase turnover (Sampson et al.

2004). Physiologically, this tonic K_{ATP} channel activation is likely to maintain a background level of channel activity that contributes a vasodilating drive to resting vascular tone. Recent evidence shows that the distance cAMP can diffuse from its site of production is severely limited by the activity of phosphodiesterases (Zaccolo & Pozzan, 2002), suggesting that downstream targets of cAMP must be reasonably close to adenylyl cyclase. The major target of cAMP, PKA, is anchored in close proximity to K_{ATP} channels through the action of an A-kinase anchoring protein (Hayabuchi et al. 2001), and we reasoned that K_{ATP} channels and their associated kinases are likely to be in the vicinity of adenylyl cyclase. In smooth muscle cells adenylyl cyclase resides primarily in small (50-100nm) cholesterol and sphingolipid-enriched invaginations of the surface membrane termed caveolae (Ostrom et al. 2002). These specialized lipid microdomains comprise approximately 20% of the smooth muscle cell's total surface area and are thought to generate subcellular signalling compartments by aggregating interacting proteins. We therefore investigated whether K_{ATP} channels are compartmentalized with adenylyl cyclase in these lipid microdomains.

Caveolar membrane fractions were isolated from rat aortic smooth muscle cell homogenates by ultracentrifugation on discontinuous sucrose gradients. Tissues were obtained from humanely killed adult male Wistar rats. Subsequent Western blot analysis showed that K_{ATP} channels localize with adenylyl cyclase, to cholesterol-rich membrane fractions containing caveolin, a structural protein found exclusively in caveolae. Additionally, an antibody against the K_{ATP} pore-forming subunit, Kir6.1 coimmunoprecipitated caveolin from arterial homogenates, suggesting that K_{ATP} channels and caveolin exist together in a complex within cells (Sampson et al. 2004). The integrity of the membrane compartments generated by caveolae seems important in maintaining normal K_{ATP} channel regulation since disruption of caveolae by the cholesterol-depleting agent, methyl-b-cyclodextrin significantly reduced the PKA-sensitive component of K_{ATP} channel current. These data indicate that tonic PKA-dependent channel activation relies on the spatial confinement of adenylyl cyclase and K_{ATP} channels.

The compartmentation of adenylyl cyclase and K_{ATP} channels presumably represents just one component of the regulatory machinery surrounding these channels, and it seems likely that larger, more elaborate signalling complexes exist within these lipid domains. The subcellular distribution of the major receptors that couple to arterial K_{ATP} channels is largely unknown, but caveolae have already been implicated as integration sites for smooth muscle Ca^{2+} signalling due to their ability to aggregate proteins involved in Ca^{2+} regulation and excitation-contraction coupling. It also seems likely that each caveola will contain a different collection of receptors and signalling proteins, which may have important implications for understanding the structural basis of K_{ATP} channel regulation.

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This work is supported by the British Heart Foundation.

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