Topical Review

Non-selective cationic channels of smooth muscle and the mammalian homologues of *Drosophila* TRP

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Throughout the body there are smooth muscle cells controlling a myriad of tubes and reservoirs. The cells show enormous diversity and complexity compounded by a plasticity that is critical in physiology and disease. Over the past quarter of a century we have seen that smooth muscle cells contain – as part of a gamut of ion-handling mechanisms – a family of cationic channels with significant permeability to calcium, potassium and sodium. Several of these channels are sensors of calcium store depletion, G-protein-coupled receptor activation, membrane stretch, intracellular Ca^{2+}, pH, phospholipid signals and other factors. Progress in understanding the channels has, however, been hampered by a paucity of specific pharmacological agents and difficulty in identifying the underlying genes. In this review we summarize current knowledge of these smooth muscle cationic channels and evaluate the hypothesis that the underlying genes are homologues of *Drosophila* TRP (transient receptor potential). Direct evidence exists for roles of TRPC1, TRPC4/5, TRPC6, TRPV2, TRPP1 and TRPP2, and more are likely to be added soon. Some of these TRP proteins respond to a multiplicity of activation signals – promiscuity of gating that could enable a variety of context-dependent functions. We would seem to be witnessing the first phase of the molecular delineation of these cationic channels, something that should prove a leap forward for strategies aimed at developing new selective pharmacological agents and understanding the activation mechanisms and functions of these channels in physiological systems.

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In this review we link two periods of discovery. The first began over 20 years ago when studies of mammalian smooth muscle tissues revealed the phenomena of store- and receptor-operated Ca^{2+}-entry pathways (Van Breemen *et al.* 1978; Bolton, 1979; Casteels & Droogmans, 1981). Over the subsequent years a large number of reports have shown these or related phenomena in a wide range of smooth muscle types. Physiological roles are suggested to include the refilling of Ca^{2+} stores, neurotransmitter and hormone-evoked Ca^{2+} entry and depolarization, and tonic cationic entry – signals that may serve to modulate myogenic tone, stimulate contraction and modulate basic cellular systems such as those controlling proliferation, death, protein trafficking and gene transcription. There are implications for disease processes including those relating to blood pressure control, smooth muscle growth, blood vessel wall thinning, subarachnoid haemorrhage, asthma, premature labour, unstable bladder, lymphatic flow, and gastrointestinal motility.

The second period of discovery began roughly 15 years later with the emergence of at least 20 mammalian homologues of the *Drosophila* transient receptor potential (*TRP*) gene (Fig. 1) (Zhu *et al.* 1995; Wes *et al.* 1995; Minke & Cook, 2002; Montell *et al.* 2002; Vennekens *et al.* 2002; Zitt *et al.* 2002; Birnbaumer *et al.* 2003; Clapham, 2003; Alexander *et al.* 2004). Several of the smooth muscle non-selective cationic channels are clearly not encoded by *TRP* genes – the P2X1 ligand-gated ion channel (Vial & Evans, 2002), the channel carrying the hyperpolarization-activated cationic current (I_f or I_h) which is presumably encoded by a *HCNx* gene (Greenwood & Prestwich, 2002), and the inositol trisphosphate (IP_3) and ryanodine receptors

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(Sanders, 2001; Fill & Copello, 2002). However, for many other non-selective cationic channels in smooth muscle there is reason to consider a *TRP* connection. First, heterologous expression of *TRP* genes induces cationic channel activity with strong permeability to K^+ and Na^+, and in most cases Ca^{2+} too. These TRP proteins are likely to be the ion pore components of the activity because they have structural homology to voltage-gated ion channels such as the *Shaker* K^+ channel, which definitively carries ions (Fig. 2). Second, expression of at least six of the *TRP* genes has been commonly reported in smooth muscle tissues, and expression of others is indicated (Fig. 3). *TRPM7* expression is said to be ubiquitous and necessary for cell survival (Nadler *et al.* 2001; Schmitz *et al.* 2003). Third, there are some general similarities in the properties of over-expressed *TRP* genes and the smooth muscle channels in question. These include non-selective cationic permeability and Ca^{2+} permeability, similarity in unitary conductance, and pharmacology including sensitivity to SKF96365 and 2-APB, and (in most cases) resistance to conventional Ca^{2+} antagonists (Fig. 4). There is also modest or weak voltage-gated activation, and a common – but by no means exclusive – 'double-rectifying' characteristic in the current–voltage-relationships (Clapham, 2003).

Categorization as a family of TRP proteins may be taken as indicative of a high degree of similarity but there is considerable diversity in sequence and function. There is commonality in hydrophobicity profiles (e.g. Fig. 2) and in many, but not all, cases there is close amino acid sequence similarity in and immediately distal to the putative sixth membrane segment (Fig. 1). The distal element may contain a clear TRP box, a signature sequence highly conserved in *Drosophila* TRP and its canonical gene products the TRPC subfamily of vertebrate TRPs. The putative fourth membrane segment (S4) has only weak similarity to the voltage sensor of *Shaker*, and the K^+ selectivity filter is absent.

Studies of native cationic channels in smooth muscle

Activation by store-depletion protocols – 'SOCs'. It is now almost a quarter of a century since studies of smooth muscle and secretory gland cells led to the suggestion of specific plasma membrane Ca^{2+} channels that open in response to a stimulus from depleted intracellular stores formed by smooth endoplasmic reticulum (Casteels & Droogmans, 1981; Putney, 1986; Parekh & Penner, 1997). These are the so-called capacitative Ca^{2+} entry (CCE) or store-operated channels (SOCs). What is the evidence for such a channel in smooth muscle? Studies of smooth muscle are no exception in showing that depletion of stores in Ca^{2+}-free medium substantially enhances the Ca^{2+} re-entry signal observed on re-admission of Ca^{2+} to the extracellular medium. Induction of such 'Ca^{2+} entry' has been demonstrated by numerous methods including the use of intracellular fluorescent dye to detect Ca^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+} or Na^+ entry, $^{45}Ca^{2+}$ flux measurements, smooth muscle contraction and electrical current recording (data are referenced in Fig. 4). The use of such a range of methods means technical factors will explain some differences and inconsistencies between

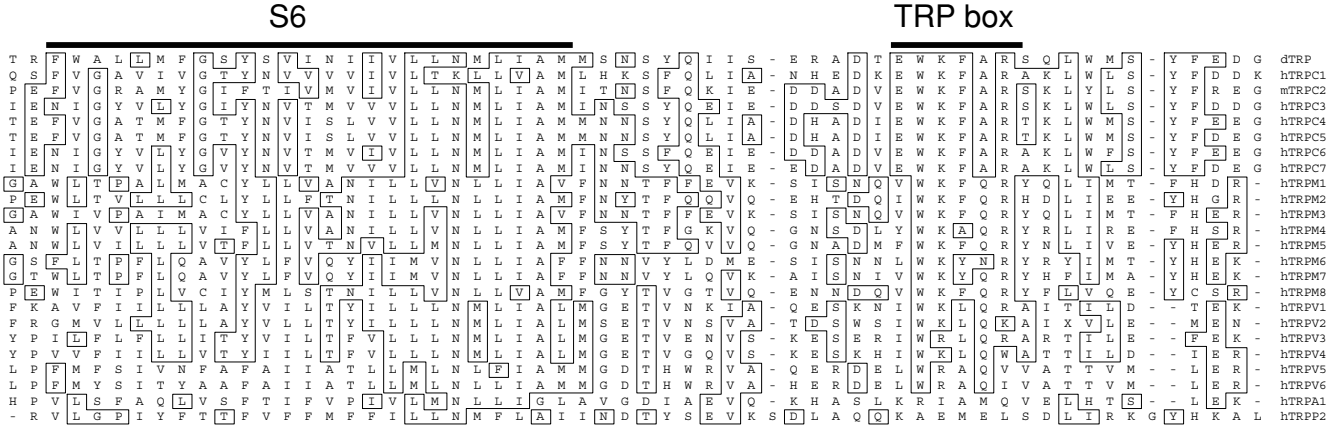

Figure 1. Members of the TRP family
Single letter-code amino acid sequence alignment (Clustal W) for one of the most highly conserved region of TRPs – the distal element of the putative sixth membrane-spanning segment (S6) and the immediate C-terminus, which contains the so-called 'TRP box'. *Drosophila* TRP (dTRP) is shown at the top. Thereafter, all sequences are human except for TRPC2, which is shown as mouse sequence because human TRPC2 would seem to be a pseudogene (i.e. not expressed as protein). TRPA (ANKTM1) and TRPP (polycystin) proteins show weak sequence similarity in this region and not all examples of these proteins are shown.

studies. For example, the reader should take due account of the fact that muscle contraction and Ca^{2+} indicator dye experiments are not direct measures of ion channel activity. Most of the ion channels under consideration have significant Na^+ as well as Ca^{2+} permeability and so indirect effects, for example via Na^+–Ca^{2+} exchange and Na^+–K^+-ATPase, are possible. Such studies should not, however, be dismissed without careful consideration of the underlying data sets because they have often correctly predicted the existence of ion channels and revealed important properties.

The available evidence supports the view that store-depletion protocols activate a particular ion channel type that is otherwise inactive. The evoked Ca^{2+} entry pathway is pharmacologically distinguishable from background pathways (Flemming *et al.* 2002, 2003) and ion channels are involved because discrete unitary current events are observed in membrane patches (Fig. 4). Unitary conductance (depending on ionic conditions) is 1.5–7 pS in three independent studies and this relatively small conductance is an emerging feature distinguishing SOCs from receptor-operated channels (Fig. 4). However, there are also similarities with receptor-operated channels (see below), and one report describes a SOC unitary conductance of 30 pS – strikingly close to that of the receptor-operated channels (Fig. 4).

The name 'SOC' implies the channels are some how controlled by signals from the stores. Proof of such a link is non-trivial and depends on an ability to make a measurement at the plasma membrane while at the same time making a highly specific manipulation of the stores. Recordings from plasma membrane can be made unequivocally but our ability to specifically manipulate the stores is open to scrutiny. Importantly, the two chemically unrelated agents thapsigargin and cyclopiazonic acid are potent inhibitors of endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and have in common the capacity to deplete stores and activate SOCs in smooth muscle (Wayman *et al.* 1996*a*, 1998, 1999; Wallace *et al.* 1999; Fig. 4). Thapsigargin does not inhibit plasma

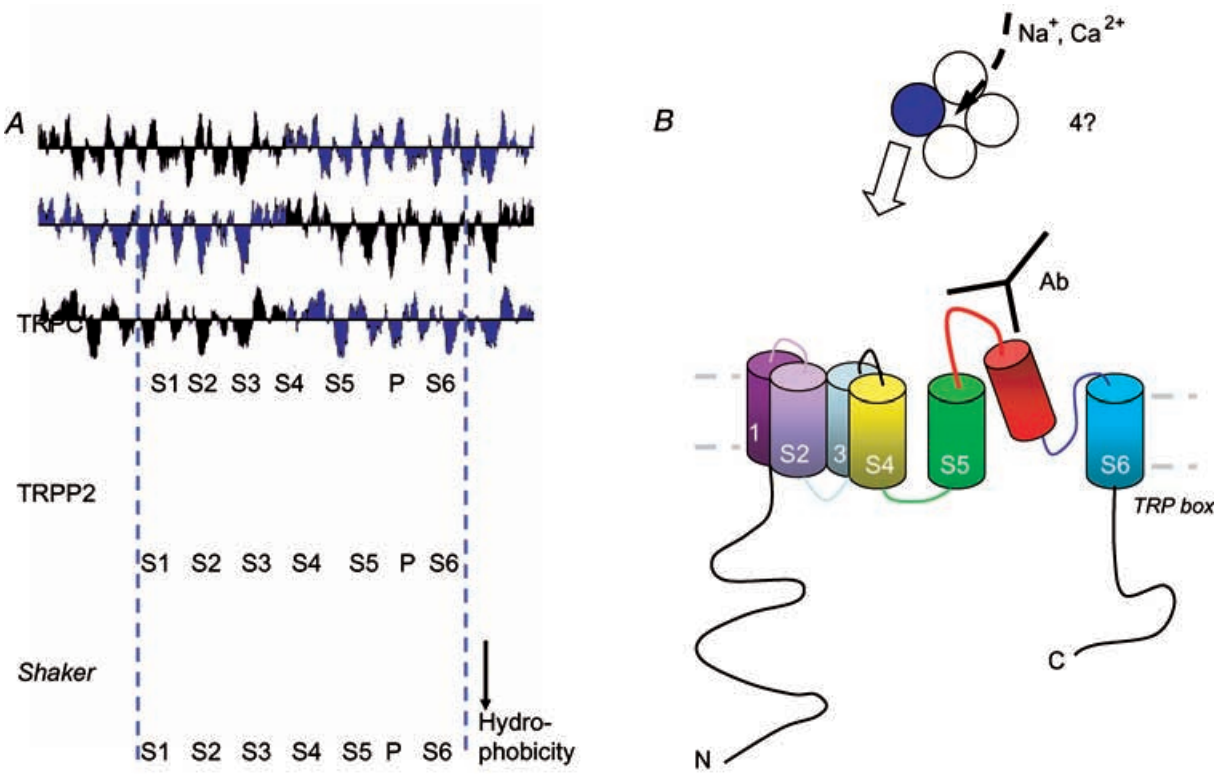

Figure 2. General structural themes of TRP proteins
A, hydropathy plots for TRPC1 (accession P48995) and TRPP2 (accession XP-011124) compared with *Shaker* voltage-gated K^+ channel (accession P08510). A theme of six membrane-spanning segments (S1-S6) and an ion selectivity filter (P-loop) is evident in each case. TRPC1 and other TRPCs have an extra hydrophobic segment towards the N-terminus. The general S1–S6 arrangement of TRPC1 has been supported by biochemical data (Dohke *et al.* 2004). All sequences have been clipped at the N (left) and C (right) termini. Hydropathy (Kyte-Doolittle) plots were created using Lasergene software (DNAStar Inc.). *B*, diagrams of the putative membrane-spanning arrangement of TRP proteins. The upper diagram is a plan view and suggests a tetrameric arrangement with ions flowing through a central pore – as for the *Shaker* K^+ channel. The lower diagram is a classical depiction of a S1–S6 protein. Extracellular targeting of antibody (Ab; not to scale) is shown for inhibition of TRPC1 function (Xu & Beech, 2001*a*; Beech *et al.* 2003; Bergdahl *et al.* 2003).

membrane Ca^{2+}-ATPases (Thastrup *et al.* 1990; Kennedy & Mangini, 1996) and mutagenesis of a SERCA-like protein in yeast stimulates Ca^{2+} entry (Locke *et al.* 2000). Thapsigargin and cyclopiazonic acid have no effect on smooth muscle SOCs in excised patches of plasma membrane where intracellular stores are likely to be absent, and so they are not direct SOC activators (Albert & Large, 2002*a*). Use of another chemical agent to deplete stores (BAPTA-AM) activates the same smooth muscle SOCs, which is considered an important result because it argues against Ca_i^{2+} elevation as the activation signal (Trepakova *et al.* 2001; Albert & Large, 2002*b*). An elegant bioassay has also shown smooth muscle SOCs in inside-out membrane patches activated by plasma membrane-permeabilized thapsigargin-treated platelets (Trepakova *et al.* 2000). These results favour the hypothesis that there is communication between depleted stores and the SOCs of the smooth muscle cell plasma membrane. Whatever the truth of this, it is indisputable that a discrete Ca^{2+} entry signal is evoked by thapsigargin or cyclopiazonic acid in, probably, every type of smooth muscle as well as many other cell types. We need to understand the molecular basis of this phenomenon if we are to know its physiological significance.

SOC signals have now been studied in a range of smooth muscle cells and different properties are apparent (Fig. 4). Although the comparisons are not within a single study, some differences are strong, giving reason to suspect multiple types of SOC (Fig. 4). This diversity may not, however, extend to the CRAC type of SOC. The Ca^{2+} release-activated channel (CRAC) of lymphocytes is one of the most studied SOCs and a highly Ca^{2+}-selective small-conductance inwardly rectifying channel (Parekh & Penner, 1997; Lewis, 1999). So far, it has not been apparent in studies of smooth muscle. Instead, a non-selective cationic SOC is most common – a channel that not only has Ca^{2+} permeability but also high Na^+ permeability and a relatively linear current–voltage relationship (Golovina *et al.* 2001; Trepakova *et al.* 2001; Albert & Large, 2002*b*).

The general mechanisms linking stores to SOC activation have been a subject of intrigue (Venkatachalam *et al.* 2002). Several features of the mechanism have emerged from studies of smooth muscle SOCs. There is an essential role for phospholipase C-γ in smooth muscle cell lines (Patterson *et al.* 2002), and in freshly isolated portal vein smooth muscle cells protein kinase C is necessary (Albert & Large, 2002*a*). Curtis *et al.* (2003) also suggest a role for protein kinase C, but an inhibitory one. The involvement of specialized subplasma membrane signalling microdomains is inferred from discrete non-contractile Ca^{2+} entry events of SOCs (Flemming *et al.* 2002) and the impact of cholesterol

Expression of TRP mRNA in smooth muscle preparations										
	C1	C2	C3	C4	C5	C6	C7	V	M	Refs
Rabbit cerebral arterioles									2	a
Rat renal resistance vessels		X					X			b
Human, rat, canine pulmonary artery	X	X	X		X		X			c
Mouse or rabbit portal vein					X		X			d
Mouse or rat aorta		X					X	2,4		e
Rat caudal artery										f
Rat cerebral artery									4	g
Human saphenous vein									2,7	h
Human, guinea-pig or rat airway								2,4		i
Human, rat myometrium / cell-line		-	X	X						j
Canine or mouse colon	X	X			X					k
Mouse stomach										l
Human oesophageal body										m
A7r5 cell line			X	X	X		X			n

Figure 3. Expression of TRP mRNA species in smooth muscle cells, cell lines and whole tissue preparations
Blue, red or black filled squares indicate mRNA was detected. Under the V and M columns, numbers indicate the subtype of TRPV or TRPM. A cross indicates mRNA could not be detected in a particular study. In some cases there is conflict between results from different research groups and so filled squares and crosses are superimposed. References: (a) Xu & Beech (2001*a*,*b*), Flemming *et al.* (2003); (b) Facemire *et al.* (2004); (c) Walker *et al.* (2001), McDaniel *et al.* (2001), Ng & Gurney (2001), Sweeney *et al.* (2002), Yu *et al.* (2003), Wang *et al.* (2004); (d) Inoue *et al.* (2001); (e) Facemire *et al.* (2003), Muraki *et al.* (2003); (f) Bergdahl *et al.* (2003); (g) Welsh *et al.* (2002), Earley *et al.* (2004), Waldron *et al.* (2004); (h) Jackson, *et al.* (2004), P. K. Jackson, B. Kumar, C. Munsch, C. D. Benham & D. J. Beech, unpublished observation; (i) Sweeney *et al.* (2002), Ong *et al.* (2003), Cloutier *et al.* (2003), Corteling *et al.* (2004), Jia *et al.* (2004); (j) Yang *et al.* (2002), Dalrymple *et al.* (2002), Babich *et al.* (2004); (k) Walker *et al.* (2001); (l) Lee *et al.* (2003); (m) Wang *et al.* (2003); (n) Jung *et al.* (2002).

depletion on SOC function, indicating association with the signalling domains of caveolae (Bergdahl *et al.* 2003). Inhibition by jasplakinolide (Patterson *et al.* 1999), an agent that stabilizes actin polymerization, indicates involvement of the cytoskeleton – suggesting a structural rearrangement near the channels either as part of an acute activation mechanism or for shuttling of key proteins in the vicinity of the SOC channels. Smooth muscle studies have also seen a major focus on the hypothesis that SOC activation depends on diffusion of a soluble factor of unknown chemical identity (called calcium influx factor, or CIF) from the emptied stores to SOCs (Trepakova *et al.* 2000; Smani *et al.* 2003). Smani *et al.* (2004) suggest CIF does not act directly on the SOCs but displaces calmodulin from iPLA2 (a calcium-independent phospholipase A2), releasing the iPLA2 from constitutive inhibition by calmodulin. A requirement for iPLA2 is indicated because antisense DNA targeted to the iPLA2 mRNA and a chemical inhibitor of iPLA2 (bromoenol lactone) inhibit SOC activation in smooth muscle cells derived from mouse aorta (Smani *et al.* 2003, 2004). iPLA2 is also activated when smooth muscle stores are depleted (Wolf *et al.* 1997). iPLA2 generates arachidonic acid and lysophospholipids, and liposomal application of lysophosphoinositol was shown to enhance SOC-like channels in inside-out membrane patches (Smani *et al.* 2004). The inference that arachidonic acid must also be generated is, however, intriguing because arachidonic acid is suggested to be an inhibitor of SOCs and a signal mediating activation of receptor-operated channels in smooth muscle (Fig. 4, and text below). Although Smani *et al.* (2004) found no effect of arachidonic acid, we observe activation of cationic channels by arachidonic acid in mouse aorta smooth muscle cells (Jackson *et al.* 2004). Therefore, there is exciting progress in the field of SOC activation but still much to be worked out. For example, how do phospholipase C and protein kinase C fit into the iPLA2 pathway? How can arachidonic acid produced by iPLA2 fail to inhibit SOCs or activate receptor-operated channels? What is CIF, how is it produced, and do smooth muscle cells produce it?

Activation by agonists at G-protein-coupled receptors – 'ROCs'. Many G-protein-coupled receptors are linked to phospholipase Cβ and so agonists at these receptors evoke IP_3 production and then Ca^{2+} release. Receptor activation may therefore lead to SOC activity. However, there are several reasons to conclude there are also separate receptor-operated channels (ROCs) – channels that require agonist binding to a G-protein-coupled receptor but that are not dependent on the Ca^{2+} release event. The first reason to conclude there are ROCs – that are not SOCs – is that store-depletion does not prevent subsequent activation of cationic channels by a receptor agonist (Wang & Large, 1991; Guibert & Beech, 1999; Zholos *et al.* 2004). Second, heparin, which blocks IP_3 receptors does not prevent ROC activation (Albert & Large, 2003*a*). Third, ROCs have a unitary conductance about 5 times larger than that of the primary SOC (Fig. 4). Fourth, SOCs and ROCs have been pharmacologically distinguished in the same cell type (Iwamuro *et al.* 1999).

ROCs have been studied extensively in several types of smooth muscle (Fig. 4). Examples receiving considerable attention include those coupled to α_1-adrenergic receptors in rabbit portal vein, M2 and M3 muscarinic receptors in guinea-pig ileum, endothelin ET-A receptors in arterial smooth muscle cells, and vasopressin V_{1a} receptors in A7r5 cells. As with SOCs, differences are apparent and so there may be multiple types of ROC (Fig. 4). For example: lanthanum activates the ileal ROC but blocks the ROC in portal vein; and LOE908 blocks ROCs in aorta-derived A7r5 cells but not in freshly isolated intra-pulmonary arterioles. However, where determined, the unitary conductances are similar and it may be premature to decide on whether 'different' ROCs are molecularly distinct entities or whether the signalling networks of different receptors confer different properties on the same channels. Nevertheless, it is striking that Iwamuro *et al.* (1998) pharmacologically distinguished two types of ROC activated by the same agonist in the same cell type.

Numerous intriguing observations have been made on the activation pathway for ROCs. Involvement of G-proteins is suggested by the common observation that agonist effects are mimicked by intracellular dialysis with the hydrolysis-resistant guanosine triphosphate (GTP) analogue GTP-γ-S (Inoue & Isenberg, 1990; Zholos & Bolton, 1994). Furthermore, pertussis toxin or intracellular dialysis with antibodies that inhibit G-protein function suppress agonist activation of ROCs (see Yan *et al.* 2003 and references therein). These studies further narrow down the type of G-protein involved to the G_i, G_o or $G_{q/11}$ α-subunits of heterotrimeric G-proteins (Kim *et al.* 1998; Wang & Kotlikoff, 2000; Lee *et al.* 2003; Yan *et al.* 2003). The chemical and widely used phospholipase C inhibitor U73122 suppresses ROC activation (Fig. 4). This may mean that the next step after G-protein activation is a classical stimulation of phospholipase C and generation of a phosphatidylinositol bisphosphate (PIP_2) product that activates the ROCs. Consistent with this hypothesis, the portal vein ROC is activated by exogenous diacyglycerol analogues – an effect that is facilitated by inositol phosphates (Helliwell & Large, 1997; Albert & Large, 2003*a*). Diacyglycerol activation of protein kinase C is required in some but not other blood vessels (Kitamura *et al.* 1992; Helliwell & Large, 1997; Slish *et al.* 2002). In contrast, the ileal ROC is not affected by diacylglycerol or inositol phosphates,

even though U73122 inhibits its activation (Zholos *et al.* 2004). Given the widespread effects of PIP_2 on ion channels (Hilgemann *et al.* 2001; Runnels *et al.* 2002), such an apparent anomaly might be explained if a fall in PIP_2 levels is a significant regulatory factor, or if a non-enzymatic function of phospholipase C is involved. Additional possibilities have emerged from studies of ROC activation by vasopressin in A7r5 cells where arachidonic acid produced by diacylglycerol lipase is suggested to stimulate nitric oxide synthase (NOS), producing nitric oxide, which then activates ROCs (Moneer & Taylor, 2002; Moneer *et al.* 2003). Although there is support for part of this hypothesis in a recent study of 5HT-activated ROCs in intrapulmonary arterioles, the expression of NOS in physiological contractile smooth muscle cells is controversial (Guibert *et al.* 2002, 2004). Not reviewed in detail here are important regulatory effects of Ca^{2+}, Mg^{2+}, H^+ and tyrosine phosphorylation (Zholos & Bolton, 1995, 1997; Helliwell & Large, 1998; Albert *et al.* 2001; Large, 2002).

Activation by membrane stretch – 'SACs'. Responses to membrane-stretch are one of the key elements in muscle tone regulation. Such responses involve sustained membrane depolarization that is resistant to conventional Ca^{2+} antagonists and due to opening of non-selective cationic channels that we refer to as stretch-activated channels (SACs). In cell-attached and inside-out patch recording modes, membrane-stretch applied through the recording pipette activates cationic channels in toad stomach, pig and human coronary artery, rat and hamster mesenteric artery, rabbit pulmonary and coronary artery, and guinea-pig urinary bladder (Kirber *et al.* 1988; Davis *et al.* 1992; Wellner & Isenberg, 1993*a*,*b*; Ohya *et al.* 1998; Wu *et al.* 2003; Park *et al.* 2003). SACs are blocked by gadolinium, unitary conductance ranges from 27 to 40 pS (charge carried by monovalent ions), and several other general properties are similar to those of ROCs (Fig. 4). In whole-cell recordings, application of longitudinal cell-stretch or cell swelling by pressure on the patch pipette or hypotonic bath solution evokes Ca^{2+}-permeable cationic channel activity in coronary artery and urinary bladder (Davis *et al.* 1992; Wellner & Isenberg, 1994). In smooth muscle the mechanisms coupling membrane stretch to channel opening seem likely to involve bioactive intermediates rather than direct mechanical activation because activation takes as long as one second – much slower than the submillisecond response time of mechano-gated channels in the fly (Walker *et al.* 2000; Gillespie & Walker, 2001). Consistent with such a hypothesis is the blocking effect of U73122, indicating a role for a signalling cascade involving phospholipase C (Fig. 4).

Figure 4. Properties of some relevant non-selective cationic channel signals in smooth muscle
Colour coding: green, permeability; red, block; grey, no effect; blue, stimulation. 'X' indicates a weak effect, a mixed effect depending on the specific agent tested, or a conflict in data from different laboratories (see specific references for details). Data sets are grouped according to SOC (store-operated channel), ROC (receptor-operated channel), SAC (stretch-activated channel), CAC (Ca^{2+}-activated channel), LAC (lipid-activated channel), BC (background channel). Asterisk indicates the possibility of a molecularly distinct subset of this channel type within one type of smooth muscle. References: (a) Loutzenhiser & Loutzenhiser (2000), Potocnik & Hill (2001), Curtis & Scholfield (2001), Guibert *et al.* (2002), Fellner & Arendshorst (2002), Flemming *et al.* (2002, 2003), Curtis *et al.* (2003); (b) Karaki *et al.* (1979), Tosun *et al.* (1998), Samain *et al.* (1999), Walter *et al.* (2000), Tanaka *et al.* (2000), Trepakova *et al.* (2000, 2001); (c) Hughes & Schachter (1994), Broad *et al.* (1999), Iwamura *et al.* (1999), Patterson *et al.* (1999, 2002), Moneer & Taylor (2002); (d) Casteels & Droogmans (1981), Golovina (1999), Doi *et al.* (2000), Golovina *et al.* (2001), Ng & Gurney (2001), Wilson *et al.* (2002), Kang *et al.* (2003); (e) Weirich *et al.* (2001); (f) Arnon *et al.* (2000); (g) Dreja *et al.* (2001); (h) Albert & Large (2002*a*,*b*); (i) Lee *et al.* (2002); (j) Ohta *et al.* (2000); (k) Wang *et al.* (2003); (l) Ito *et al.* (2002), Sweeney *et al.* (2002); (m) Shlykov *et al.* (2003); (n) Patterson *et al.* (1999); (o) Broad *et al.* (1996); (p) Curtis & Scholfield (2001); (q) Stepien & Marche (2000); (r) Matsuoka *et al.* (1997); (s) Wayman *et al.* (1996*a*,*b*, 1998, 1999), Wallace *et al.* (1999), Gibson *et al.* (2001), McFadzean & Gibson (2002); (t) Guibert *et al.* (2004); (u) Snetkov *et al.* (2003); (v) Byrne & Large (1988), Amédee *et al.* 1990, Wang & Large (1991), Kitamura *et al.* (1992), Inoue & Kuriyama (1993), Oike *et al.* (1993), Helliwell & Large (1997, 1998), Aromolaran & Large (1999), Albert *et al.* (2001), Inoue *et al.* (2001), Large (2002), Albert & Large (2003*a*); (w) Van Renterghem & Lazdunski (1994), Nakajima *et al.* (1996), Iwasawa *et al.* (1997), Minowa *et al.* (1997), Iwamuro *et al.* (1998, 1999), Kawanabe *et al.* (2001, 2002), Jung *et al.* (2002), Moneer & Taylor (2002), Moneer *et al.* (2003); (x) Murray & Kotlikoff (1991), Wang & Kotlikoff (2000), Oonuma *et al.* (2000); (y) Amédee *et al.* (1990), Wang *et al.* (1993), Large (2002); (z) Welsh & Brayden (2001); (aa) Kim *et al.* (1995, 1997, 1998), Kang *et al.* (2001), Lee *et al.* (2003), So *et al.* (2003); (ab) Vogalis & Sanders (1990; (ac) Bayguinov *et al.* (2001); (ad) Benham *et al.* (1985), Inoue *et al.* (1987, 1994, 1995, 1998), Inoue & Isenberg (1990*a*,*b*), Inoue (1991), Chen *et al.* (1993), Zholos & Bolton (1994, 1995), Bakhramov (1995), Shi *et al.* (2003), Yan *et al.* (2003). (ae) Guibert *et al.* (2004). (af) Minowa *et al.* (1997), Iwamuro *et al.* (1998, 1999), Kawanabe *et al.* (2001, 2002); (ag) Muraki *et al.* (2003; (ah) Welsh *et al.* (2000), Slish *et al.* (2002; (ai) Wu & Davis (2001), Park *et al.* (2003), Wu *et al.* (2003; (aj) Wellner & Isenberg (1993*a*,*b*, 1994), Kushida *et al.* (2001); (ak) Loirand *et al.* (1991); (al) Jabr *et al.* (2000); (am) Terasawa *et al.* (2002); (an) Albert *et al.* (2003*b*); (ao) Bae *et al.* (1999); (ap) Zakharov *et al.* (1999, 2003); (aq) Hughes & Schachter (1994); (ar) Miyoshi *et al.* (2004); (as) Thorneloe & Nelson (2004).

Linking native cationic channels to TRP homologues

The potential for a link between native channels and TRP homologues is addressed in three stages. First, we have asked if there is evidence for expression of the protein and whether it is at the plasma membrane. Second, we have only focused on the protein if there is 'a direct link' – by which we mean the use of a TRP-specific tool to study the smooth muscle cationic channel. This might mean the use of gene disruption, RNA targeting with antisense DNA, or a specific blocking antibody. Some conventional pharmacological agents inhibit TRP function but none have the specificity that can provide a direct link. Lastly, we compare the properties of the native cationic channel with channel activity generated by heterologously over-expressed cDNA.

TRPC1 and TRPC4/5. The *TRPC1* gene is widely expressed as mRNA and protein, including in a range of smooth muscle cell types (Fig. 3) (Xu & Beech, 2001*a*; Dalrymple *et al.* 2002; Ong *et al.* 2002; Sweeney *et al.* 2002). The protein is localized to the plasma membrane, although intracellular localization is also evident (Xu & Beech, 2001*a*; Dalrymple *et al.* 2002). Glycosylation of TRPC1 has been suggested (Ong *et al.* 2002) but the consensus N-linked glycosylation site is weak and between S5 and S6 (Fig. 1). We have not been able to detect glycosylation of TRPC1 (S. Z. Xu & D. J. Beech, unpublished obsevation). The apparent molecular mass is about 90 kDa (Beech *et al.* 2003). Splice variants have been reported but the functional significance is unknown (Beech *et al.* 2003).

Three studies provide evidence of a direct link between TRPC1 and Ca^{2+}-permeable cationic channels of vascular smooth muscle. In each case, TRPC1 has only been linked to Ca^{2+} entry associated with store depletion. In two of the studies a functional anti-TRPC1 antibody was used to specifically inhibit TRPC1 (Xu & Beech, 2001*a*; Bergdahl *et al.* 2003). This antibody is targeted to the putative outer vestibule of the ion-pore (Fig. 2), acts extracellularly, and is specific for a 90 kDa protein in vascular smooth muscle samples. In one of the studies the antibody was shown to partially inhibit the Ca^{2+} re-entry signal in store-depleted smooth muscle cells of rabbit arterioles, without effect on background Ca^{2+} entry (Xu & Beech, 2001*a*). In the other, on rat caudal artery, the antibody inhibited contractions that had a pharmacological profile like that of store-operated Ca^{2+} entry (Bergdahl *et al.* 2003). Antisense DNA targeted to TRPC1-encoding mRNA has also been applied to human pulmonary artery smooth muscle cells cultured to passages 4–6 (Sweeney *et al.* 2002). After 24 h treatment there was a reduction in RNA, as well as protein detected with anti-TRPC1 antibody. The labelled protein was large, at 220 kDa, and suggested to be a dimer of TRPC1 – an aggregating effect that is common with membrane proteins. The functional effect of the antisense DNA was a 50–70% reduction in the amplitude of the non-selective cationic current evoked by store depletion. In support of a role for TRPC1 in the smooth muscle SOC phenomenon, over-expression of TRPC1 enhanced pulmonary artery contraction evoked by cyclopiazonic acid but not 40 mM K^+ (Kunichika *et al.* 2004).

We cannot be sure of the properties of TRPC1 because it is difficult to obtain substantial functional signals over background in response to over-expression of TRPC1 alone (reviewed in Beech *et al.* 2003). It also may not be relevant to focus on such properties because TRPC1 probably does not act physiologically on its own (i.e. as a homomeric channel assembly). There is ample evidence that TRPC1 forms heteromeric arrangements, particularly with TRPC4 and 5, but also involving TRPC3 and TRPP2 (Tsiokas *et al.* 1999; Lintschinger *et al.* 2000; Strübing *et al.* 2001, 2003; Goel *et al.* 2002; Hofmann *et al.* 2002; Xu *et al.* 2002) (Fig. 3). For example, in HEK-293 cells, TRPC1 trafficked poorly to the plasma membrane unless coexpressed with TRPC4β (Hofmann *et al.* 2002). The over-expression studies yielding a functional TRPC1 signal may have been positive because there were sufficient native TRPs to support function. If we nevertheless assume these were pure TRPC1 signals, TRPC1 would seem to be a non-selective cationic channel subunit with permeability to Ca^{2+} and barium but not strontium or *N*-methyl-D-glucamine ($NMDG^+$). It is blocked by gadolinium, lanthanum (IC_{50} 1–10 μM), 80 μM 2-aminoethoxydiphenyl borate (2-APB), 20 μM xestospongin C or high concentrations of extracellular Ca^{2+}, but not by 1 mM zinc (reviewed in Beech *et al.* 2003). Negative feedback results from Ca^{2+} binding to calmodulin and there is activation by the calmodulin inhibitor 1 μM calmidazolium (Delmas *et al.* 2002; Singh *et al.* 2002). The current–voltage relationship has only mild rectification over a wide voltage range. Stimulation of TRPC1 by diacylglycerols was apparent in the absence but not presence of extracellular Ca^{2+} (Lintschinger *et al.* 2000; Delmas *et al.* 2002), a weak effect when compared with those on TRPC3, TRPC6 and TRPC7 (Hofmann *et al.* 1999; Okada *et al.* 1999). Regulation of TRPC1 by a range of accessory proteins has been proposed (see Fig. 6 for references).

Although there are studies both in favour and against a role for TRPC1 in SOC phenomena we favour the conclusion that TRPC1 is a pore-forming subunit of the *in situ* non-selective cationic SOC of vascular smooth muscle cells (Xu & Beech, 2001*a*; Beech *et al.* 2003; Bergdahl *et al.* 2003). The failure of many investigators to find function when over-expressing cDNA encoding TRPC1 may mean that it cannot function alone. In this regard TRPC5 is of interest because TRPC1–TRPC5 heteromultimers are functional and have a conductance of about 5 pS (Strübing *et al.* 2001) – close to the unitary conductance of smooth muscle SOC (Fig. 4). TRPC5 was initially indicated to be

primarily expressed in brain and some studies of smooth muscle have not detected TRPC5 mRNA (Fig. 3). However, an increasing number of reports reveal TRPC5 mRNA in smooth muscle and we have developed several anti-TRPC5 antibodies that show TRPC5 protein is present (Fig. 3) (Xu *et al.* 2002). Furthermore, an anti-TRPC5 antibody inhibits Ca^{2+} entry evoked by store depletion in arterioles (Beech *et al.* 2004), suggesting TRPC1 and TRPC5 are involved in smooth muscle SOCs (Figs 5 and 6). TRPC5 over-expressed alone has properties similar to those of the muscarinic cationic ROC of gastric smooth muscle (Lee *et al.* 2003) and thus TRPC5 may have multiple functional roles (Zeng *et al.* 2004). TRPC4 is the most closely related protein to TRPC5, has similar properties in over-expression systems, and can form a complex with TRPC1 (Strübing *et al.* 2001; Plant & Schaefer, 2003). The mRNA encoding TRPC4 is detected in smooth muscle preparations (Fig. 3) and antisense DNA treatment of cultured mesangial cells inhibits Ca^{2+} entry associated with store depletion (Wang *et al.* 2004). Although the smooth muscle α-actin marker is not expressed in normal adult mesangial cells, it appears in glomerular disease and cell culture (Stephenson *et al.* 1998). Therefore, TRPC4 and TRPC5 are candidates as subunits assisting TRPC1 in its function as an element of the SOC phenomenon in smooth muscle (Fig. 6).

TRPP1 and TRPP2. TRPP1 and TRPP2 are also called polycystin 1 and polycystin 2, or PKD1 and PKD2, or PC1 and PC2. They have general structural similarity to *Shaker* K^+ channel and TRPC1 (Fig. 2), although they lack a distinct TRP box and high sequence identity with TRPCs (Fig. 1). Recent classifications include them in the TRP superfamily (Birnbaumer *et al.* 2003; Clapham, 2003). The C-terminal portion of TRPP1 also has similarity but is a much larger protein with an additional N-terminal feature. The names of these proteins derive from their role in polycystic kidney disease. However, the phenotypes resulting from TRPP mutations in humans and knock-out mice are complex and vascular abnormality is striking – particularly aneurysmal disease. Furthermore, there is good agreement that TRPP1 and TRPP2 are expressed as protein in smooth muscle cells (Griffin *et al.* 1997; Kim *et al.* 2000; Gao *et al.* 2004). Expression is observed in major arteries such as the aorta, and in intracranial arteries, the afferent arteriole, and many more (Boulter *et al.* 2001; Torres *et al.* 2001). TRPP1 is about 400 kDa and several forms may be expressed, especially early in development. TRPP2 is about 110 kDa and is consistently expressed through development and in the adult animal (Qian *et al.* 2003*b*). Glycosylation studies suggest TRPP1 is expressed in intracellular and plasma membranes, where as TRPP2 seems mostly in intracellular membranes (Qian *et al.* 2003*b*). Gold-labelling electron microscopy studies localize TRPP1 to the plasma membrane and both proteins near to dense plaques, which are associated with linkage of thin filaments to the elastic laminae (Qian *et al.* 2003*b*).

Evidence of a direct link of TRPPs to smooth muscle cationic channels comes partly from murine *TRPP1* gene disruption experiments in which the homozygotes die at embryonic day 15.5 from massive haemorrhage, which is suggested to be explained by abnormality in the functions of vascular endothelial and smooth muscle cells (Kim *et al.* 2000). There are, however, more data focused specifically on smooth muscle: TRPP2+/− mice have abnormalities of Ca^{2+} handling in smooth muscle cells freshly isolated from the aorta. Resting $[Ca^{2+}]_i$, Ca^{2+} release evoked by caffeine and thapsigargin, and Ca^{2+} re-entry in store-depleted cells were suppressed by 13–28% with statistical P values < 0.008 (Qian *et al.* 2003*a*). Qian *et al.* (2003*a*) suggest resting $[Ca^{2+}]_i$ and Ca^{2+} release are suppressed because store-operated Ca^{2+} entry is compromised. TRPP2 is a protein partner of TRPC1 (Tsiokas *et al.* 1999) (Figs 5 and 6) and, although the functional consequence of this interaction is unknown, it is notable that both proteins have impact on the store-operated Ca^{2+} channel signal

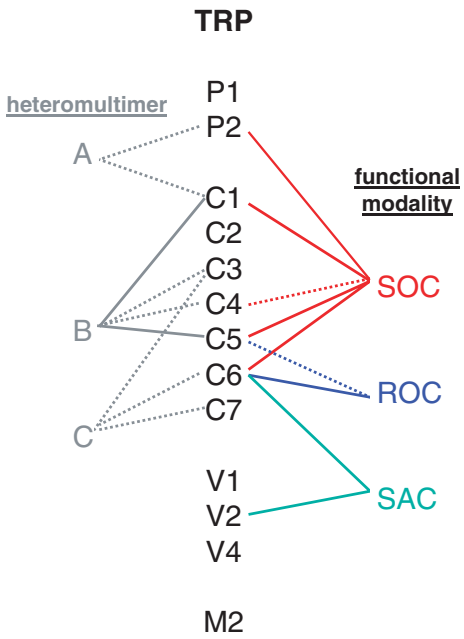

Figure 5. Schematic diagram to illustrate how diversity might arise in TRP-dependent non-selective cationic channels
Three origins of diversity are proposed: (i) expression of multiple independent *TRP* genes, each with distinct properties; (ii) heteromultimerization of different TRP proteins within a tetrameric complex, with diversity arising due to differing expression levels of component TRP subunits; (iii) 'functional modality', by which we mean that each TRP may have the capability to be activated by several different signals (also referred to as versatility or promiscuity of gating, or multiplicity of activation). Continuous lines indicated that there is direct evidence in a smooth muscle preparation. Dotted lines indicated speculation based on work on other cell types or where evidence is not direct. See text for references and supporting information.

of vascular smooth muscle cells. There is an apparent conflict between the intracellular localization of TRPP2 and plasma membrane localization of TRPC1 (Xu & Beech, 2001; Qian *et al.* 2003*b*). However, the cellular localization of these proteins is actively debated, there is TRPC1 inside cells as well as the membrane, and TRP proteins may shuttle to the membrane in response to stimuli or associated proteins (Hanaoka *et al.* 2000; Koulen *et al.* 2002; Beech *et al.* 2003; Cayouette *et al.* 2004). Further evidence of a link between TRPP2 and native cationic channels comes from a study of visceral smooth muscle in *Drosophila* in which loss-of-function TRPP2 mutations severely reduced smooth muscle contractile function (Gao *et al.* 2004). Contractility was restored by expressing wild-type TRPP2.

The precise properties of heterologously expressed TRPP proteins are unclear in part because of differences between several published studies. However, there is agreement that they are non-selective cationic channels that pass Ca^{2+}. They are weakly selective with evidence of currents carried by the large cation $NMDG^{+}$, magnesium, and even anions. Hanaoka *et al.* (2000) only found membrane currents when TRPP1 and TRPP2 were coexpressed. The current reversed near 0 mV and the current–voltage relationship was near-linear, or slightly outwardly rectifying. There was about 50% block by 50 μM La^{3+} and no effect of 50 μM niflumic acid. Ion channel function of TRPP2 was observed in lipid bilayers and when expressed in insect sf9 cells (Gonzalez-Perrett *et al.* 2001). There were multiple conductance states and amiloride

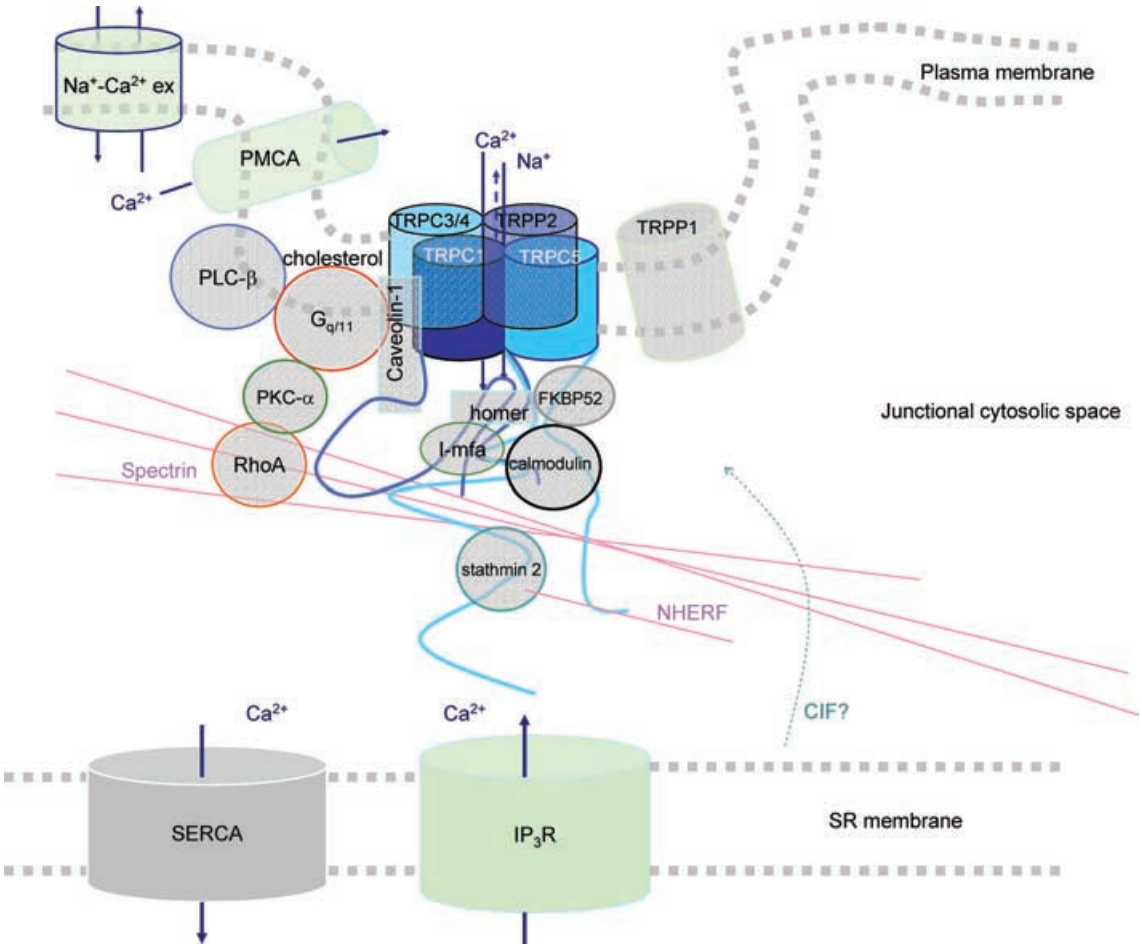

Figure 6. Putative local protein and signalling network associated with TRPC1
One possible heteromultimer is indicated. Homer is suggested to dissociate from inositol trisphosphate receptor (IP_3R) when stores are depleted (Yuan *et al.* 2003). There is evidence CIF acts on SOCs but there is no direct information for TRP (Trepakova *et al.* 2000; see text). References: Tsiokas *et al.* (1999), Lintschinger *et al.* (2000), Lockwich *et al.* (2000), Tang *et al.* (2000, 2001), Trepakova *et al.* (2000), Strübing *et al.* (2001), Hofmann *et al.* (2002), Kunzelmann-Marche *et al.* (2002), Rosado *et al.* (2002), Singh *et al.* (2002), Vaca & Sampieri (2002), Beech *et al.* (2003), Bergdahl *et al.* (2003), Brazer *et al.* (2003), Cioffi *et al.* (2003), Greka *et al.* (2003), Ma *et al.* (2003), Mehta *et al.* (2003), Boulter *et al.* (2001), Qian *et al.* (2003*b*), Yuan *et al.* (2003), Ahmmed *et al.* (2004), Sinkins *et al.* (2004).

was inhibitory (IC_{50} 79 μM). Heterologous expression of TRPP2 alone seems not to lead to ion channel function in the plasma membrane of mammalian cells but function has been observed in recordings from porcine kidney cell endoplasmic reticulum-derived vesicles (Koulen *et al.* 2002). There was marked inward rectification, unitary conductance of 90–114 pS, permeability to Ca^{2+}, Ba^{2+} and Mg^{2+}, and activation by cytosolic Ca^{2+} (0.1–100 μM). Ca^{2+} release evoked by vasopressin was markedly enhanced by TRPP2, consistent with TRPP2 acting as a Ca^{2+}-activated Ca^{2+} release channel. This effect was inhibited by 50–100 μM 2-APB. The C-terminal element of TRPP1 functions in the plasma membrane when over-expressed on its own in *Xenopus* oocytes (Vandorpe *et al.* 2001). It is sensitive to block by lanthanum, gadolinium, zinc, SKF96365 or amiloride, but not the chloride channel inhibitors DIDS or niflumic acid. Therefore, TRPP proteins are Ca^{2+}-permeable cationic channels with a general pharmacology similar to other TRP proteins. They may function in intracellular and plasma membranes.

TRPC6. There is *TRPC6* gene expression in smooth muscle as mRNA and protein (Fig. 3) (Inoue *et al.* 2001; Dalrymple *et al.* 2002; Welsh *et al.* 2002; Yu *et al.* 2003). The protein is localized near to the plasma membrane in freshly isolated portal vein cells but is more perinuclear in cultured cells (Inoue *et al.* 2001). Consistent with plasma membrane localization there is N-linked glycosylation. Deglycosylation shifted the apparent molecular mass from about 130–110 kDa (Yu *et al.* 2003), close to the predicted molecular mass of full-length TRPC6 and the mass of over-expressed full-length TRPC6 (Zhang & Saffen, 2001). Two splice variants of TRPC6 have been described, one of which appears non-functional as a protein (Zhang & Saffen, 2001; Jung *et al.* 2003). The splicing events result in deletions and a reduction in mass by about 10 or 20 kDa. Other deletion splices are evident in the sequence databases. Translation of these RNAs to form smaller TRPC6 proteins has not been evident in studies of smooth muscle.

Three studies provide evidence of a direct link between TRPC6 and cationic channels of vascular smooth muscle. In each case, isolated cells (Inoue *et al.* 2001; Yu *et al.* 2003) or tissues (Welsh *et al.* 2002) were incubated with antisense DNA targeted to *TRPC6* mRNA for 12–24 h (Yu *et al.* 2003) or 3–5 days (Inoue *et al.* 2001; Welsh *et al.* 2002). In each case, TRPC6 protein levels were shown to be decreased either by immunostaining or Western blot and evidence of specificity was provided. Strikingly, however, the functional properties of the TRPC6-related signal were different in each study. Inoue *et al.* (2001) described activation of cationic channels by the α_1-adrenoceptor agonist phenylephrine in rabbit portal vein smooth muscle cells. Welsh *et al.* (2002) described activation of cationic channels by the hypo-osmotic stress in rat cerebral artery smooth muscle cells. Yu *et al.* (2002) described activation of cationic channels by a store-depletion protocol in rat pulmonary artery smooth muscle cells at passage 3–6. One conclusion from this could be that receptor-, stretch- and store-operated properties are encoded by a single gene (Fig. 5). There may be multiple activators of TRPC6 or each stimulus may elevate a common second messenger substance. A candidate second messenger is diacylglycerol because it activates TRPC6 and is elevated by α_1-adrenoceptor activation or membrane stretch. Protein kinase C activation by diacylglycerol is also proposed as a stimulus for activation of store-operated channels (Albert & Large, 2002*a*). If a common messenger is involved, it is unlikely to be the whole story. Each TRPC6-related signal had a distinct current–voltage relationship – from outwardly rectifying, to double rectifying, to inwardly rectifying. Such differences may be explained in part by heteromultimerization of TRPC6 with other TRPC proteins. Strong evidence exists for association of TRPC6 with TRPC3 or TRPC7, and both may be expressed in smooth muscle (Fig. 3). We also cannot exclude that there are differences between TRPC6 from different species. Human and mouse TRPC6 diverge at 70 amino acids – almost exclusively in the crucial N- and C-termini.

Numerous studies include descriptions of the properties of heterologously expressed mouse, rat or human TRPC6, and there are few discrepancies between the results (Boulay *et al.* 1997; Hofmann *et al.* 1999, 2002; Inoue *et al.* 2001; Zhang & Saffen, 2001; Boulay, 2002; Basora *et al.* 2003; Dietrich *et al.* 2003; Jung *et al.* 2003; Lee *et al.* 2003; Estacion *et al.* 2004; Hisatsune *et al.* 2004). The unitary conductance of the channel is between 28 and 46 pS and the single channel unitary current–voltage relationship is linear. The reversal potential is close to 0 mV, there is no permeability to the large cation $NMDG^+$, and the estimated ratio of Ca^{2+} to Na^+ permeability is 4.5. Stimulation of the channels has occurred in response to G-protein-coupled receptor activation, intracellular GTP-γ-S or aluminium fluoride, relatively high concentrations of diacylglycerol and related compounds (EC_{50} for activation by 1-oleoyl-2-acetyl-*sn*-glycerol, 117 μM; Hofmann *et al.* 1999), the arachidonic acid metabolite 20-HETE, extracellular 1 mM Ca^{2+}, or 100 μM flufenamate, but not to intracellular IP_3, thapsigargin, phorbol esters, intracellular PIP_2, ionomycin or intracellular alkalinization. Basora *et al.* (2003) found no effect of flufenamate. TRPC6 signals have been inhibited by 10 μM U73122, 2 μM calmidazolium, 250 μM cadmium, 4–6 μM lanthanum, 2 μM gadolinium, 4 μM SKF96365 or 130 μM amiloride. Rat TRPC6A was inhibited by phorbol ester, an effect prevented by protein kinase C inhibition (Zhang & Saffen, 2001). Boulay (2002) found an inhibitory effect of coexpressing a Ca^{2+}-insensitive

mutant of calmodulin. Glycosylation at the putative second extracellular loop suppresses constitutive activity of TRPC6 (Dietrich *et al.* 2003). Fyn-mediated tyrosine phosphorylation stimulates TRPC6 (Hisatsune *et al.* 2004). Most often there is a double rectifying current–voltage relationship characteristic of TRPC channels, but two studies show only mild outward or inward rectification (Boulay *et al.* 1997; Basora *et al.* 2003). Both of the latter studies were on the same stable cell-line expressing mouse TRPC6 with an HA tag. The commonly observed outward rectification may result from an increase in open probability at positive voltages, perhaps reflecting a mild intrinsic voltage-sensing mechanism akin to that in voltage-gated potassium channels. Single channel opening events of TRPC6 are very brief (Hofmann *et al.* 1999; Jung *et al.* 2003).

Inoue *et al.* (2001) provided, in addition to antisense DNA results, an exemplary comparison of the properties of mouse TRPC6 and the α_1-adrenoceptor-linked cationic channel of rabbit portal vein smooth muscle cells. In all comparisons there were marked similarities – further strengthening the conclusion that TRPC6 is a critical element of this endogenous cationic channel and that it may operate in portal vein myocytes as a homomultimeric assembly. There are no reports of stretch- or store-operated properties of over-expressed TRPC6.

TRPV2. TRPV2, a member of the vanilloid receptor TRP subfamily, is mainly expressed in sensory organs, but is also widely distributed as mRNA and protein in non-sensory organs including vascular smooth muscles (Fig. 3) (O'Neil & Brown, 2003). Although intracellular localization of the protein is evident, TRPV2 has been suggested to localize to the plasma membrane in the presence of growth factors (Kanzaki *et al.* 1999). Glycosylation of TRPV2 has been suggested (Kanzaki *et al.* 1999), although the molecular mass of TRPV2 in cardiac and aortic smooth muscle is 85–90 kDa – close to the predicted mass of non-glycosylated full-length TRPV2 (~86 kDa) (Iwata *et al.* 2003; K. Muraki & Y. Imaizumi, unpublished observation).

Striking new evidence has emerged for a role of TRPV2 in vascular smooth muscle (Fig. 4) (Muraki *et al.* 2003). In mouse aortic smooth muscle cells, cell-swelling caused by hypotonic solution activated non-selective cationic channels and elevated $[Ca^{2+}]_i$. These responses were inhibited by ruthenium red, a blocker of TRPV channels. Removal of external Ca^{2+} abolished elevation of $[Ca^{2+}]_i$ evoked by hypotonic solution, suggesting stimulation of Ca^{2+} entry rather than Ca^{2+} release. TRPC6 expression was weak, but TRPV2 mRNA was detected and TRPV2 immunoreactivity was evident in single mouse aortic, mesenteric and basilar arterial smooth muscle cells. Treatment of mouse aorta with TRPV2 antisense DNA reduced the amount of TRPV2 protein and suppressed hypotonic stimulation-induced activation of cationic channels or the associated elevation of $[Ca^{2+}]_i$. Therefore, it would seem that TRPV2, as well as TRPC6, can function as a stretch-activated channel in vascular smooth muscle. Studies of intact arteries will be required for an understanding of the relative physiological significance of different TRP channels in responses to pressure, stretch and cell-swelling.

There are few studies on the properties of over-expressed TRPV2. However, consistent with the above studies on native smooth muscle cells, CHO-K1 (Chinese hamster ovary) cells over-expressing mouse TRPV2 exhibited non-selective channels that were stimulated by membrane stretch through the recording pipette or superfusion with hypotonic solution (Muraki *et al.* 2003). Moreover, stretch of the cells on an elastic silicone membrane elevated $[Ca^{2+}]_i$. Mechanisms involved in opening of TRPV2 by membrane-stretch have not been determined. However, deletion of ankyrin repeats, which are present in the N-terminal region of TRPV, abolished heat-activation of TRPV1 and TRPV4 (Schumacher *et al.* 2000; Watanabe *et al.* 2002). Since the ankyrin repeats interact with certain cytoskeletal proteins, this region of TRPV2 might be important for acceptance of applied mechanical stimuli. However, compared with the delay to opening of fly mechanoreceptor channels, TRPV2, TRPV4 and TRPC6 are slowly activating, suggesting 'mechano-modulation' rather than direct 'mechano-gating' (Gillespie & Walker, 2001). One hypothesis to explain this is that cell swelling or membrane stretch produce an endogenous ligand – perhaps a fatty acid – which activates TRPV2. Metabolites of arachidonic acid and diacylglycerol might modulate TRPV2, like they do TRPV4 and TRPC6 (Hofmann *et al.* 1999; Watanabe *et al.* 2003). Micro-domains comprising channels, receptors, lipids, enzymes and dynamically controlled lipids could be sensors of local membrane deformation.

Molecular basis of functional heterogeneity

A review of the many studies of non-selective cationic channels in smooth muscle reveals differences between pharmacological, regulatory and biophysical properties within or across smooth muscle types (Fig. 4). What is the basis of such diversity? Some ideas are summarized in Figs 5 and 6.

One explanation might be that there is expression of many *TRP* genes. Indeed, we already know that over half of the *TRP* genes are expressed as mRNA (Fig. 3), and protein evidence exists for some of them (see above). Added diversity may arise from heteromultimeric assembles, which are evident for TRPC1, {C3}, C4, C5 and P2, and TRPC3, C6 and C7 (Tsiokas *et al.* 1999; Hofmann *et al.* 2002; Strübing *et al.* 2003) (Fig. 5). Different smooth muscles may have

different quantities of TRP proteins, leading to different stoichiometries of heteromultimers and tendencies to homomultimerization. For example, enhanced expression of TRPC3 may confer greater constitutive activity on a heteromultimer involving TRPC6, generating background cationic channels (Dietrich *et al.* 2003; Albert *et al.* 2003*b*). There is some evidence for differential TRP mRNA expression in smooth muscle (Fig. 3 and references therein).

There may be an additional mechanism for diversity. In Fig. 4 it is striking that single channel recordings have tended to reveal two primary unitary conductances: about 5 and 25 pS. Furthermore, one of the noticeable outcomes in reviewing TRPC6 in connection with smooth muscle is that three studies suggest three apparently separate functions. Also, single channel events just like those of the store-operated channel have been activated by noradrenaline in excised patches and thus seemingly independently of store-depletion (Albert & Large, 2002*a*). These are just a few emerging examples of the behaviour of TRP-like cationic channels. We suggest that such observations may be explained by different functional modalities of the same TRP protein. That is, the same TRP protein can have, for example, receptor- and stretch-operated properties, etc. (Fig. 5). Such versatility, or promiscuity, of gating is evident for over-expressed TRPC5 and TRPV4 (Vriens *et al.* 2004; Zeng *et al.* 2004). Cellular context may shift the emphasis from one gating mode to another.

Diversity in function can also result from differential expression of protein partners with a channel subunit. In the *Drosophila* TRP field the concept of a signalplex has emerged (Li & Montell, 2000). Similarly, a model is emerging for a signalplex associated with mammalian TRP channels. Figure 6 shows an example for TRPC1. Intriguingly, although many of the associated proteins have been proposed from studies outside the smooth muscle field, the majority are proteins already well-associated with smooth muscle function. Protein kinase C-α and caveolin-1 are examples fitting well with functional studies of smooth muscle SOCs (Albert & Large, 2002*a*; Bergdahl *et al.* 2003).

Roles of TRP proteins and non-selective cationic channels in tissues

Although we can state that a TRP protein forms a cationic channel in a heterologous system and may contribute, for example, to receptor-, stretch- and store-operated mechanisms *in situ*, these are not statements on the importance in whole physiological or pathological systems. However, information is emerging on the importance of these mechanisms: deletion of TRPP genes causes aneurysmal disease, perhaps suggesting a role in the secretory function of smooth muscle (Kim *et al.* 2000). Block of TRPC1 inhibits endothelin-evoked contraction in rat caudal artery (Bergdahl *et al.* 2003). Antisense DNA targeted to TRPC1 or TRPC6 inhibits proliferation of pulmonary artery smooth muscle cells in culture (Sweeney *et al.* 2002; Yu *et al.* 2003). Suppression of TRPC6 expression in cerebral artery inhibits myogenic tone (Welsh *et al.* 2002). The chemical agent LOE908, a commonly used inhibitor of receptor-operated channels, suppresses vasospasm and delays expansion of ischaemic damage in animal models of stroke (Hoehn-Berlage *et al.* 1997; Li *et al.* 1999; Tatlisumak *et al.* 2000; Kawanabe *et al.* 2003). Therefore, there would seem to be widespread roles of TRP proteins in the vascular and associated systems. Roles in other types of smooth muscle should be delineated soon.

TRPing along, or TRPing up?

If we are TRPing up and *TRP* genes do not explain the non-selective cationic channels of smooth muscle, where else might we look to solve these channels? CD20 protein is suggested to contribute to store-operated properties in myoblasts (Ju *et al.* 2003; Li *et al.* 2003). Although it is proposed as the first non-TRP candidate for SOCs, in predicted structure it shows a relationship to the S5–S6 region of TRPs. To our knowledge, CD20 is not expressed in smooth muscle. A few SOC signals are inhibited by classical Ca^{2+} antagonists (Fig. 4), leading some to propose the existence of truncated L-type voltage-gated Ca^{2+} channel α_{1C}-subunit with SOC properties (Stokes *et al.* 2004). However, these would not be non-selective cationic channels. There are many other types of non-selective cationic channel, including IP_3 receptors and P2X1 receptors, which have unitary conductance of about 75 and 25 pS, respectively. If we accept the concept of versatility of gating, should these channels be candidates? Signalling cascades of receptors and membrane deformation are complex and multifactorial, SERCA inhibition may profoundly impact on protein trafficking and local secretory mechanisms, leading to diverse effects on numerous proteins. A range of ion handling mechanisms may be influenced by such stimuli.

Nevertheless, in our opinion, the evidence is in favour of the conclusion that TRP proteins are the molecular basis of a significant number of the previously unsolved non-selective cationic channels of smooth muscle. The evidence is best for an association of specific TRP proteins with store-operated, receptor-operated and stretch-activated channels, but there are other candidates too, including background cationic channels and non-selective channels activated by lysophosphatidylcholine, Ca^{2+}, and alkaline pH (Loirand

et al. 1991; Zakharov *et al.* 1999, 2003; Jabr *et al.* 2000; Terasawa *et al.* 2002; Poteser *et al.* 2003) (Fig. 4). Since gating versatility is an emerging feature of TRP channels (Vriens *et al.* 2004; Zeng *et al.* 2004) these other effects may simply be features of TRP channels already known to be expressed and functional in smooth muscle. Versatility of gating is an interesting concept because it may enable TRP channels to act as physiological integrators or be a mechanism for increasing diversity of proteins without evolution of new genes. Alternatively, other *TRP* genes may be responsible for the diversity. For example, *TRPM* gene expression has been detected at mRNA level in smooth muscle (Fig. 3) and thus may underlie additional cationic channels. Recent reports suggest roles for TRPC3, TRPV4, TRPM2 and TRPM4 (Earley *et al.* 2004; Jackson *et al.* 2004; Jia *et al.* 2004; Waldron *et al.* 2004). TRPM7 is also a reasonable candidate given its proposed ubiquitous expression and our detection of mRNA in saphenous vein; it may have a role as a Mg^{2+} channel (Schmitz *et al.* 2003). A difficult but crucial element of any such study is proof of native protein expression at the plasma membrane. Once this is done it is a reasonable expectation that the protein will do something akin to that observed in heterologous expression systems, i.e. have cationic channel function. We need more TRP protein data, and more direct evidence of links with native cationic channels. Despite such hurdles, the future for studies of mammalian homologues of *Drosophila* TRP in smooth muscle looks promising. We might finally have found the genes that encode the calcium entry mechanisms initially brought to our attention a quarter of a century ago by the seminal works of Bolton, Casteels, Droogmans, van Breemen and their colleagues.

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