Topical Review

Multiple activation mechanisms of store-operated TRPC channels in smooth muscle cells

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Store-operated channels (SOCs) are plasma membrane Ca^{2+}-permeable cation channels which are activated by agents that deplete intracellular Ca^{2+} stores. In smooth muscle SOCs are involved in contraction, gene expression, cell growth and proliferation. Single channel recording has demonstrated that SOCs with different biophysical properties are expressed in smooth muscle indicating diverse molecular identities. Moreover it is apparent that several gating mechanisms including calmodulin, protein kinase C and lysophospholipids are involved in SOC activation. Evidence is accumulating that TRPC proteins are important components of SOCs in smooth muscle. More recently Orai and STIM proteins have been proposed to underlie the well-described calcium-release-activated current (I_{CRAC}) in non-excitable cells but at present there is little information on the role of Orai and STIM proteins in smooth muscle. In addition it is likely that different TRPC subunits coassemble as heterotetrameric structures to form smooth muscle SOCs. In this brief review we summarize the diverse properties and gating mechanisms of SOCs in smooth muscle. We propose that the heterogeneity of the properties of these conductances in smooth muscle results from the formation of heterotetrameric TRPC structures in different smooth muscle preparations.

(Received 1 June 2007; accepted after revision 2 July 2007; first published online 5 July 2007)
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In smooth muscle cells agonists of G-protein-coupled receptors (GPCRs) produce contraction by increasing cytosolic calcium concentration ($[Ca^{2+}]_c$). This increase in $[Ca^{2+}]_c$ results from release of Ca^{2+} ions from intracellular stores, mainly the sarcoplasmic reticulum (SR), and influx of Ca^{2+} ions from the extracellular medium. A component of this Ca^{2+} influx is mediated by voltage-dependent Ca^{2+} channels (VDCCs) but a significant contribution of Ca^{2+} influx occurs independently of VDCCs (Large, 2002). Moreover the contribution of Ca^{2+} influx through voltage-independent channels varies markedly according to the type of smooth muscle. This is part of the explanation underlying the observation that blockers of L-type Ca^{2+} channels are more effective in blocking Ca^{2+} influx and contraction of some smooth muscle preparations (e.g. resistance blood vessels and intestine) compared to other tissues (e.g. tracheal smooth muscle and many large blood vessels; see Bolton, 1979). Thus, during cell stimulation in the former preparations most of the Ca^{2+} influx occurs through VDCCs while in the latter tissues Ca^{2+} enters the cell mainly through voltage-independent Ca^{2+} channels. There has been much research recently into voltage-independent Ca^{2+} channels in smooth muscle not only because of their physiological importance but also because these pathways represent realistic targets for therapeutic intervention in important diseases involving excessive smooth muscle contraction such as hypertension and asthma.

Similar to many other cell types there is significant evidence for the expression of Ca^{2+}-permeable cation channels in smooth muscle which are classified as receptor-operated (ROCs) and store-operated channels (SOCs; see reviews by Large, 2002; McFadzean & Gibson, 2002; Albert & Large, 2003; Beech *et al.* 2004). Moreover there is accumulating evidence that members of the canonical subgroup of transient receptor potential (TRPC) channels constitute both ROCs and SOCs in smooth muscle. ROCs are activated in response to cell surface receptor (usually GPCR) stimulation which is independent of depletion of internal Ca^{2+} stores. For example, the native TRPC6-like channel in rabbit portal vein is activated by noradrenaline acting on α-adrenoceptors but this channel is not stimulated by agents that directly release Ca^{2+} from the SR (Wang & Large, 1991; Inoue *et al.* 2001). Generally ROCs are activated by GPCRs coupled to phospholipase C (PLC) although in rabbit ear artery myocytes ROCs

 DOI: 10.1113/jphysiol.2007.137802

Table 1. Comparison of the biophysical properties of store-operated channel current (I_{soc}) in vascular myocytes with calcium-release-activated current (I_{CRAC}) in non-excitable cells

	Cell type	Single channel conductance	P_{Ca}/P_{Na}	Activation mechanisms	Molecular components of channel
I_{soc}	Aorta[a,b]	~3 pS	1 : 1	CIF, iPLA$_2$ and LPLs	—
	Portal vein[c,d,e]	~2 pS	50 : 1	PKC, CaM	TRPC1/C5[f]
	Mesenteric artery[g]	ditto	ditto	PKC	TRPC1[g]/C5[f]
	Pulmonary artery[h]	~5 pS (20 mM $[Ca^{2+}]_o$)	—	—	—
	Mesangial cells[i,j,k]	~3 pS	—	PKC	TRPC4
I_{crac}[l,m]	Mast cells RBL-1/2H3 Jurkat T cells	~0.02 pS (110 mM $[Ca^{2+}]_o$)	1000 : 1	STIM1	Orai1

—, information not available; CIF, calcium influx factor; iPLA$_2$, calcium-independent phospholipase A$_2$; LPLs, lysophospholipids; PKC, protein kinase C; CaM, calmodulin. [a]Trepakova *et al.* (2001); [b]Smani *et al.* (2004); [c]Albert & Large (2002*a*); [d]Albert & Large (2002*b*); [e]Albert *et al.* (2006*b*); [f]authors' unpublished data, see Fig. 4; [g]Saleh *et al.* (2006); [h]Golovina *et al.* (2001); [i]Ma *et al.* (2000); [j]Ma *et al.* (2002); [k]Wang *et al.* (2004); [l]Parekh & Putney (2005); [m]Lewis, 2007). Unless indicated in parentheses, the conductance values were obtained in 1.5 mM $[Ca^{2+}]_o$.

may be constitutively driven by phospholipase D (Albert *et al.* 2005). In contrast SOCs are plasmalemmal ion channels which are proposed to be stimulated by depletion of internal Ca^{2+} stores. The strict definition of a SOC is a channel which is activated by a decrease in the Ca^{2+} concentration within the SR ($[Ca^{2+}]_{SR}$, or endoplasmic reticulum, ER) and not by the subsequent rise (or reduction) in $[Ca^{2+}]_c$ (Parekh & Putney, 2005). Further it is sometimes stated that SOCs are not activated by second messengers generated by PLC (Lewis, 2007). An important question is whether a single cellular mechanism activates SOCs and several hypotheses have been tested over the years (see Parekh & Putney, 2005 for a detailed review). In the present review we will briefly summarize the properties of SOCs in smooth muscle where they have been studied at the single channel level. It is intended to highlight the fact that SOCs in smooth muscle have diverse biophysical properties and multiple activation mechanisms and to indicate possible molecular explanations for these different characteristics.

Single channel properties of SOCs in smooth muscle

In physiological conditions SOCs are evoked by stimulation of plasmalemmal GPCRs (e.g. α-adrenoceptors in vascular smooth muscle) coupled to PLC with subsequent formation of inositol-1,4,5-trisphosphate (IP_3) which causes release of Ca^{2+} ions from the SR and subsequent opening of SOCs. However, receptor agonists are rarely used to study SOCs because they are expected to simultaneously activate ROCs which would complicate interpretation of data from such experiments. Consequently the usual method to record SOC activity in isolation is to study responses evoked by the selective SR Ca^{2+}-ATPase (SERCA) inhibitors thapsigargin and cyclopiazonic acid (CPA). In smooth muscle the first recording of a store-operated conductance was measured with whole-cell recording from a single mouse anococcygeus myocyte treated with CPA (Wayman *et al.* 1996). This type of experiment has been carried out in several laboratories (see Albert & Large, 2003) but often the $[Ca^{2+}]_c$ is not buffered to a fixed concentration and it is possible that the observed current is therefore evoked by changes in $[Ca^{2+}]_c$.

The gold standard method of recording SOC activity is to measure whole-cell currents evoked by depleting intracellular Ca^{2+} stores, e.g. with SERCA inhibitors, while clamping the $[Ca^{2+}]_c$ to approximately resting levels with high (10 mM) concentrations of a calcium chelator such as BAPTA or EGTA. It is important to point out that these conditions are unlikely to be achieved physiologically and cell stimulation will normally be accompanied by an increase in $[Ca^{2+}]_c$. Nevertheless such experiments have demonstrated that CPA induces a whole-cell current in freshly dispersed rabbit portal vein myocytes (Albert & Large, 2002*a*; Liu *et al.* 2005*b*) and human airway smooth muscle (Peel *et al.* 2006). In these conditions it is assumed that the channel underlying the whole-cell current is a SOC according to the strict definition of SOC activation.

However, there is a great advantage in recording SOC activity at the single channel level because it is possible to be more confident of studying a single molecular mechanism which may not be the case with whole-cell recording or measurement of $[Ca^{2+}]_c$ with dyes. There have been several studies which demonstrate that single Ca^{2+}-permeable cation channels with unitary conductances of 2–5 pS can be recorded in the cell-attached configuration in isolated smooth muscle cells in response to application of CPA or thapsigargin (Table 1, mesangial cells are included because they possess a similar phenotype to smooth muscle).

In contrast the widely studied Ca^{2+}-release-activated current (I_{CRAC}) has not been studied at the single channel level but a unitary conductance has been estimated to be approximately 0.02 pS from stationary noise analysis (Table 1). The disparity in unitary conductances highlights important differences between SOCs in smooth muscle and I_{CRAC} in non-excitable cells and indicates that these channels are likely to possess different molecular structures (see later).

Some of these studies were carried out with cell-attached recording but this configuration is limited in that it is likely that SERCA inhibitors raise $[Ca^{2+}]_c$ which may be the stimulus to activate the assumed SOCs rather than depletion of $[Ca^{2+}]_{SR}$. However, it has been shown that the cell-permeable Ca^{2+} chelator BAPTA-AM, which will decrease $[Ca^{2+}]_c$ and hence passively deplete $[Ca^{2+}]_{SR}$, also activates single channels with identical properties to those induced by thapsigargin in aortic myocytes (Trepakova *et al.* 2001) and CPA in rabbit portal vein (Albert & Large, 2002*a*) and mesenteric artery (Saleh *et al.* 2006). Hence it seemed reasonable to conclude that the single channels recorded with the cell-attached configuration were genuine SOCs.

Diverse biophysical properties and activation mechanisms of SOCs in vascular smooth muscle

Parekh & Putney (2005) have discussed the variety of store-operated calcium entry mechanisms in different cell types but it also seems that there are at least two classes of SOCs in smooth muscle. This issue is described in detail by Albert & Large (2003) but Table 1 illustrates the two main different biophysical characteristics of SOCs in rabbit aortic myocytes compared to portal vein/mesenteric artery myocytes. The unitary conductances are similar in all three preparations in 1.5 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) but they have significantly different Ca^{2+} permeabilities. SOCs in portal vein/mesenteric artery myocytes are approximately 50 times more permeable to Ca^{2+} ions than the SOC in aortic myocytes when estimated from reversal potentials in different $[Ca^{2+}]_o$ (Table 1).

It is apparent that the relative Ca^{2+} permeability of SOCs in smooth muscle is considerably smaller than I_{CRAC} (see Table 1) and thus SOCs in smooth muscle are non-selective cation channels with a degree of Ca^{2+} permeability. It seems that I_{CRAC} is well suited for refilling intracellular Ca^{2+} stores whereas in smooth muscle SOCs may also represent a depolarizing mechanism as well as a Ca^{2+} influx pathway. These differences in Ca^{2+} permeability between SOCs in smooth muscle and I_{CRAC} in non-excitable cells provide further evidence that these channels are likely to possess different molecular structures (see later).

In addition there appears to be at least two activation mechanisms of SOCs in smooth muscle. In aortic myocytes it has been proposed that depletion of $[Ca^{2+}]_{SR}$ releases a calcium influx factor (CIF) from the stores which displaces calmodulin (CaM) bound to membrane-delimited Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) which produces lysophospholipids to open SOCs (Smani *et al.* 2004). This represents a classical store-dependent activation mechanism. In contrast, in rabbit portal vein and mesenteric artery myocytes, SOCs evoked by both CPA and BAPTA-AM are almost completely blocked by inhibitors of protein kinase C (PKC) indicating a pivotal role for this kinase in SOC activation (Albert & Large, 2002*b*; Saleh *et al.* 2006). Moreover the diacylglycerol (DAG) analogue 1-oleoyl-*sn*-glycerol (OAG), and phorbol esters which stimulate PKC, also induced SOC activity in portal vein and mesenteric artery myocytes (Albert & Large, 2002*b*; Saleh *et al.* 2006). However, it is not clear how a reduction of $[Ca^{2+}]_{SR}$ leads to activation of PKC and subsequent SOC opening. Therefore the SOCs in aorta *versus* portal vein/mesenteric artery differ in both biophysical properties and gating mechanisms.

Store-independent activation of SOCs in rabbit portal vein myocytes

A significant observation was that the sympathetic neurotransmitter noradrenaline activated SOCs in outside-out patches of portal vein myocytes in which CPA had no effect (Fig. 1*A*). These channels had identical characteristics to SOCs evoked by CPA and BAPTA-AM in cell-attached patches which were blocked by PKC inhibitors and therefore this noradrenaline-induced activity can be termed store-independent activation of SOCs (Albert & Large, 2002*b*). Presumably noradrenaline activates membrane-delimited PKC which is mediated by the production of DAG. In addition the phosphatase inhibitor calyculin A also evoked SOCs (Fig. 1*B*) in outside-out patches. This result not only supports the notion that a kinase may be involved in SOC activation but also suggests that there is a constitutively active phosphorylating pathway working independently of intracellular stores which can open SOCs. This store-independent pathway is in contrast to the established definition of SOC activation.

Figure 2*A*, *B* and *D* illustrates SOC activation in inside-out patches of portal vein myocytes by the phorbol ester PDBu, a PKC catalytic subunit and inhibition by a PKA catalytic subunit. These data reinforce the idea that store-independent activation of SOCs is mediated by PKC and also demonstrate that the channels may be inhibited by protein kinase A (PKA)-dependent mechanisms (Liu *et al.* 2005*b*). Therefore the channels can be stimulated and inhibited in excised membrane patches where there appears to be no functional Ca^{2+} stores and hence channel activity is not related to $[Ca^{2+}]_{SR}$. It was shown

that β-adrenoceptor stimulation inhibits SOC activity via PKA in portal vein myocytes and therefore this mechanism may contribute to the vasodilatation mediated by β-adrenoceptors (Liu *et al.* 2005*b*). An interesting finding was that although IP_3 applied on its own to the cytoplasmic surface did not induce SOC activity it did markedly potentiate the probability of channel opening of SOCs evoked by PKC catalytic subunit (Fig. 2*D*) and by PDBu, CPA and BAPTA-AM (Liu *et al.* 2005*a*). Therefore the intracellular mediator which releases Ca^{2+} from the SR also potentiates the influx mechanism for refilling the store. Previously one hypothesis for SOC activation is the conformational coupling mechanism whereby IP_3 receptors in the SR couple to the plasmalemmal SOC on depletion of $[Ca^{2+}]_{SR}$ to cause channel opening (Parekh & Putney, 2005). However, in our experiments there is no evidence for the presence of SR in inside-out patches (due to lack of effect of CPA) and therefore this effect may be due to IP_3 itself acting on or close to the SOC. Application of the PKA inhibitor H-89 also induced channel activity (Fig. 2*C*), which supports the hypothesis that there is a tonically active stimulation mechanism which is independent of $[Ca^{2+}]_{SR}$ and also that there is a constitutive inhibitory pathway involving PKA.

It should also be noted that in human glomerular mesangial cells epidermal growth factor-activated SOCs have been shown to be mediated via a store-independent mechanism which does not involve IP_3 receptors or release of Ca^{2+} ions from internal Ca^{2+} stores (Li *et al.* 2004).

Calmodulin stimulates SOCs in rabbit portal vein myocytes

The hypothesis that there is a constitutive driver causing SOC opening, which is counteracted by constitutive inhibitory PKA stimulation, provoked us to investigate whether calmodulin (CaM) stimulates SOCs as this Ca^{2+}-binding protein is known to modulate many types of ion channel, including Ca^{2+}-permeable channels. Application of 100 nM CaM to the intracellular surface of an inside-out patch stimulates a 2 pS channel in portal vein myocytes (Fig. 3*Aa* and *b*) which has identical properties to SOCs (Albert *et al.* 2006*a*). This stimulation of SOCs by CaM is Ca^{2+} dependent and SOCs are stimulated in 1 nM $[Ca^{2+}]_c$ with a maximal effect around 100 nM $[Ca^{2+}]_c$. In higher $[Ca^{2+}]_c$ the excitatory effects of CaM decrease (Fig. 3*Ac*). It should be noted that direct application of

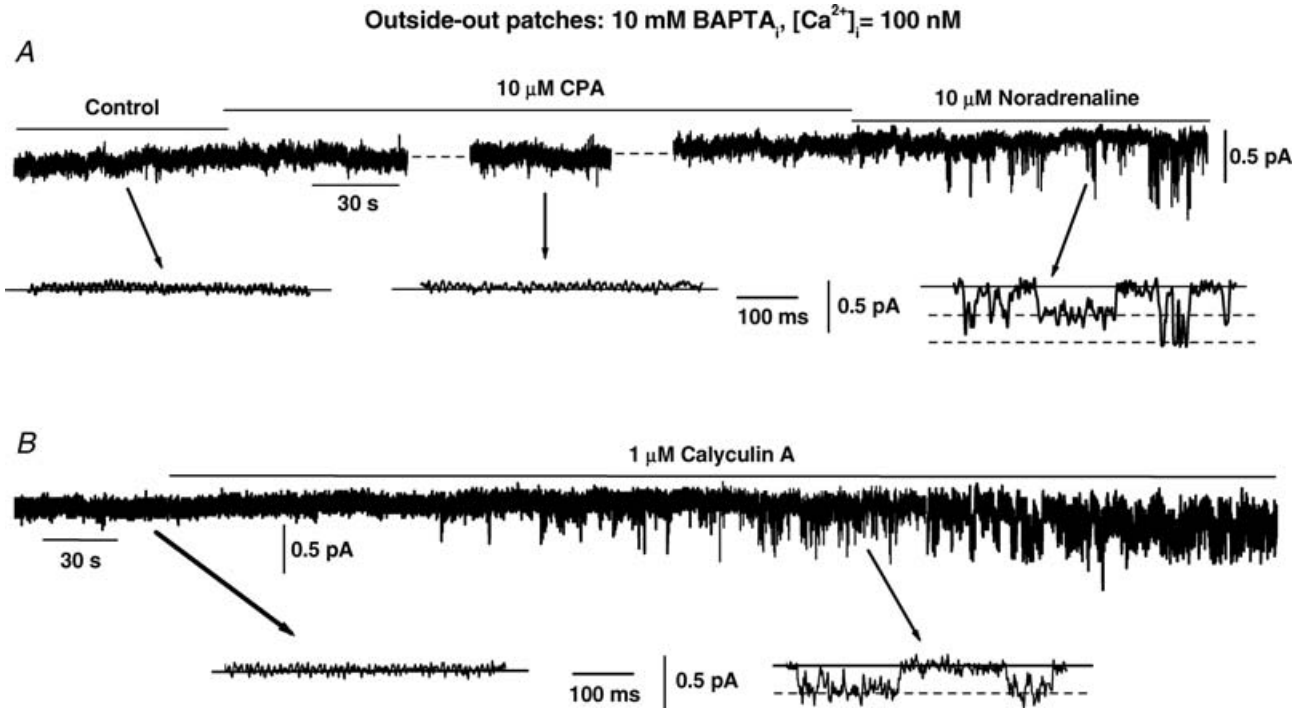

Figure 1. Store-independent activation of store-operated channel (SOC) activity by noradrenaline and calyculin A in rabbit portal vein myocytes
A, bath application of cyclopiazonic acid (CPA) did not evoke SOC activity whereas subsequent bath application of noradrenaline did induce SOC activity in an outside-out patch held at −70 mV. *B*, bath application of calyculin A stimulated SOC activity in a quiescent outside-out patch at −70 mV. Figures reproduced from Albert & Large (2002*b*).

Ca^{2+} ions (up to 2 μM) alone to the cytoplasmic surface of inside-out patches never evokes these channels (Albert & Large, 2002*b*).

Further support for a role of CaM in activating SOCs comes from the observation that the CaM antagonist calmidazolium (CMZ) completely inhibits SOC activity evoked by BAPTA-AM (Fig. 3*B*). However, the involvement of CaM seems to be complicated since application of CMZ to quiescent cells initially stimulates SOCs which were subsequently inhibited with continued application of CMZ (Fig. 3*C*). The initial stimulation by CMZ seems to result from removal of an inhibitory action of CaM kinase II since addition of CaM kinase II inhibitors produce sustained SOC activation (Fig. 3*D*). Therefore CaM appears to have dual actions: it directly activates SOCs but may also produce inhibition by stimulating CaM kinase II and these dual actions are illustrated in Fig. 3*E* where addition of purified CaM kinase II inhibits SOCs evoked by CaM (see Albert *et al.* 2006*a* for full details). Therefore in rabbit portal vein myocytes both PKC and CaM appear to have pivotal roles in SOC activation although the precise conditions for the involvement of PKC and CaM remain to be elucidated.

In summary, different gating mechanisms exist for SOCs in different smooth muscle types (aorta *versus* portal vein/mesenteric artery in rabbit) and, in addition, studies in the portal vein indicate that SOCs can be activated by diverse pathways which do not require depletion of intracellular Ca^{2+} stores with one pathway involving activation by PKC and the other utilizing CaM. Two noteworthy points are that, first, CaM evokes SOC activity in physiological concentrations ($[CaM]_c$ in many cell types is about 1–10 μM; Saimi & Kung, 2002) and in $[Ca^{2+}]_c$ between 1 and 100 nM providing a possible basis for a constitutive driver mechanism. Secondly, it is possible that SOCs may be activated by increasing $[Ca^{2+}]_c$ via CaM (or perhaps a Ca^{2+}-dependent PKC). Additionally SOCs may also be stimulated by a decrease in $[Ca^{2+}]_c$ due to the removal of the inhibitory influence of CaM kinase II. With respect to these points it should be remembered that in an intact cell, as when recording from a cell-attached patch, it is likely that CPA will increase $[Ca^{2+}]_c$ whereas BAPTA-AM will reduce $[Ca^{2+}]_c$. Hence the effect of these agents on $[Ca^{2+}]_c$ should always be taken into account when appraising experimental approaches used to investigate SOC activity.

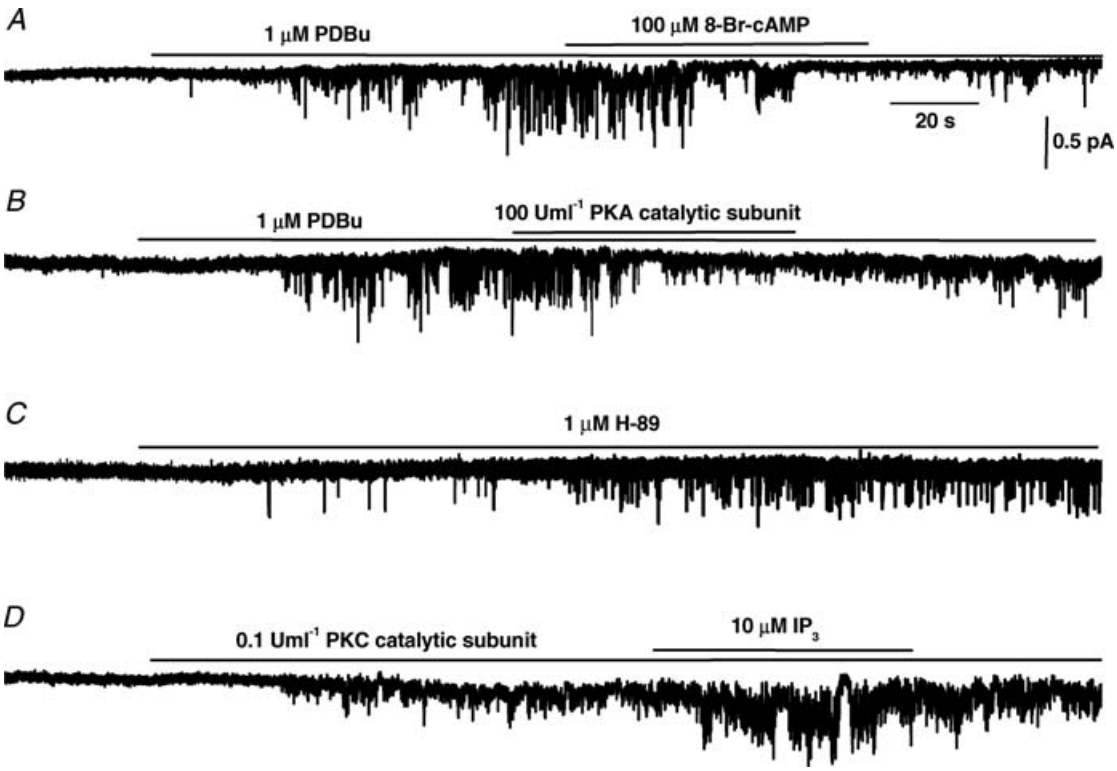

Figure 2. Role of PKC and PKA in SOC activation in rabbit portal vein myocytes
A, bath application of PDBu activated SOC activity in an inside-out patch at −80 mV which was reversibly inhibited by co-application of 8-Br-cAMP. *B* shows that PDBu-induced SOC activity is inhibited by co-application of a PKA catalytic subunit in an inside-out patch at −80 mV. *C*, bath application of H-89 induced SOC activity in a quiescent cell-attached patch at −80 mV. *D* shows that SOC activity evoked by a PKC catalytic subunit was potentiated by co-application of IP_3 in an inside-out patch at −80 mV. Figures reproduced from Liu *et al.* (2005*a*,*b*).

Molecular composition of SOCs

The discovery of mammalian homologues of the *Drosophila* transient receptor potential (TRP) gene which encode 28 non-selective cation channel proteins with varying permeabilities to Ca^{2+} ions, has lead to a plethora of studies on the possible role of TRP channels underlying SOCs. Many of these studies have focused on the canonical transient receptor potential (TRPC) subfamily which comprises seven channel proteins (TRPC1–C7, C2 is a pseudogene in humans). Over-expression studies in cultured cell lines have shown that almost all TRPC channel proteins can be activated by SERCA inhibitors and by dialysing the intracellular medium with high concentrations of Ca^{2+} chelators, which suggests that TRPCs may represent SOCs (Parekh & Putney, 2005).

An important point when considering the molecular composition of TRPCs underlying SOCs is the general opinion that the majority of functional TRPCs *in vivo* consist of different TRPC subunits associated together in complex heterotetrameric structures. A variety of techniques including co-immunoprecipitation, fluorescence resonance energy transfer (FRET) and expression of dominant-negative TRPC subunits have shown that TRPC1 can form interactions with TRPC4/C5 and that TRPC3/C6/C7 channel proteins can also interact with one another (Goel *et al.* 2002; Hofmann *et al.* 2002). Moreover in embryonic rat brain microsomes associations between TRPC1 and TRPC4/C5 and between TRPC3/C6 have also been described (Strübing *et al.* 2003). Functional studies have shown that TRPC1 and TRPC3 associate to form distinct channels in human parotid gland ductal cells (HSY) cells which were activated by store depletion using thapsigargin and also by the DAG analogue 2-acetyl-*sn*-glycerol indicating a possible store-independent activation pathway (Liu X. *et al.* 2005). In rat H19-7 hippocampal cell lines TRPC1/TRPC3 have also been proposed to mediate store-operated Ca^{2+} entry (Wu *et al.* 2004). Moreover TRPC1 and TRPC4 proteins are thought to form a heterotetrameric SOC in endothelial

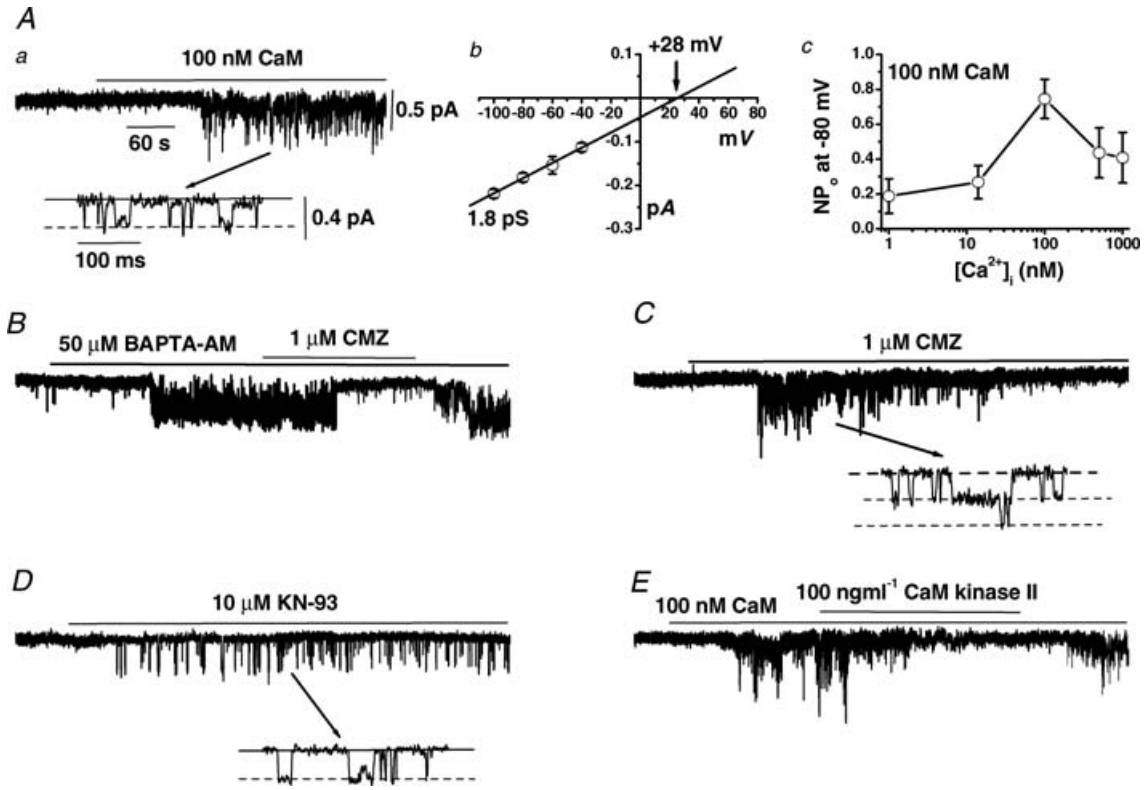

Figure 3. CaM activates SOC activity and CaM kinase II has an inhibitory effect on SOC activity in rabbit portal vein myocytes

Aa, bath application of CaM induces SOC activity in an inside-out patch at −80 mV; *b* shows that CaM-activated SOCs had a slope conductance of 1.8 pS and an extrapolated reversal potential of +28 mV; and c illustrates that CaM-evoked SOC activity is modulated by changing $[Ca^{2+}]_i$. *B* shows that BAPTA-AM-induced SOC activity was blocked by co-application of the CaM antagonist calmidazolium (CMZ) in a cell-attached patch at −80 mV. *C*, bath application of CMZ to a quiescent cell activated SOC activity that subsequently declined in a cell-attached patch at −80 mV. *D*, bath application of the CaM kinase II inhibitor KN-93 evoked sustained SOC activity in a cell-attached patch at −80 mV. *E* shows that CaM-evoked SOC activity in 100 nM $[Ca^{2+}]_i$ was reversibly inhibited by co-application of purified CaM kinase II in an inside-out patch at −80 mV. *Aa* and *b*, *B*, *C*, *D* and *E* were reproduced from Albert *et al.* (2006*b*). *Ac* is authors' previously unpublished data.

cells (Brough *et al.* 2001; Freichel *et al.* 2001; Ahmmed *et al.* 2004) and TRPC1/C3/C7 are thought to form endogenous SOCs in HEK-293 cells (Zagranichnaya *et al.* 2005). In summary, it is apparent that numerous TRPC subunits can interact in a highly complex manner to form SOCs in several cell types.

A recent advance in the understanding of the potential molecular composition of SOCs has been the discovery of two families of transmembrane proteins, STIM and Orai, which have been proposed to mediate I_{CRAC} in non-excitable cells with STIM1 acting as an ER Ca^{2+} sensor/activator of Orai1 and Orai1 constituting the CRAC channel/ion transport mechanism (see Lewis, 2007 for review and references). As there are vast differences between the biophysical properties of I_{CRAC} and many SOCs (see above and Table 1) it seems unlikely that Orai proteins alone mediate the pore-forming subunits of all SOCs. However, recent studies have suggested that STIM1 and Orai1 may interact with TRPC proteins to modify their function. Huang and colleagues showed that over-expression of the cytosolic terminus of STIM1 increased TRPC1 activity and also demonstrated that STIM1 and TRPC1 proteins can associate with one another (Huang *et al.* 2006). In addition over-expression of Orai proteins was shown to enable thapsigargin to activate TRPC3 and TRPC6 activity through a STIM1-mediated mechanism which was not present in the absence of Orai proteins (Liao *et al.* 2007). These results indicate that STIM proteins may act as store-operated regulators of SOCs and also that Orai proteins may combine with TRPCs to produce functional store-operated channels either through acting as a pore-forming subunit or as a regulatory β-subunit (Huang *et al.* 2006). STIM1 has also been shown to regulate agonist-evoked activity of all TRPC subunits, except TRPC7, through either directly binding to TRPC1, TRPC4 and TRPC5 proteins or mediating heteromultimerization of TRPC3 and TRPC6 proteins with these STIM1-binding TRPC subunits (Yuan *et al.* 2007). Importantly this study proposed a new definition of SOCs, as channels that are regulated by STIM1 and require store-depletion-mediated clustering of STIM1 for activation, and concluded that all TRPCs, except TRPC7, can function as SOCs (Yuan *et al.* 2007). Furthermore TRPC1 has been suggested to produce a ternary complex with STIM and Orai1 which is important for activating SOCs in human salivary glands (Ong *et al.* 2007).

To date there is little information on the role of STIM and Orai in smooth muscle although human airway myocytes have been shown to express STIM1/2 mRNA and siRNA targeted at STIM1 markedly reduced Ca^{2+} influx and whole-cell currents evoked by CPA (Peel *et al.* 2006) indicating a potentially important role for STIM in mediating SOCs in these myocytes. In light of the proposed roles of STIM1 in regulating TRPC activity (see above) and the increasing evidence indicating that TRPCs mediate SOCs in smooth muscle (see below) an important area of future research will be to investigate the roles/mechanisms of STIM proteins in regulating SOC activity in smooth muscle.

The diverse molecular compositions of I_{CRAC} (by Orai proteins) and SOCs (possibly a heterotetrameric TRPC structures) provides a possible explanation as to why there are many types of SOCs with different biophysical properties, permeabilites to Ca^{2+} and activation mechanisms. Furthermore this potential diversity in the make-up of SOCs poses the question against the strict definition of SOCs: is it probable that activation of these channels is governed only by a single store-operated mechanism?

Molecular identity of SOCs in vascular smooth muscle

Recently several groups using different experimental approaches have presented evidence for TRPC1 being an essential component of SOCs in smooth muscle. In rabbit cerebral artery myocytes anti-TRPC1 antibodies raised against a putative extracellular epitope of TRPC1 reduced thapsigargin-induced Ca^{2+} influx (Xu & Beech, 2001) and in pulmonary artery myocytes inhibition of endogenous TRPC1 expression by specific antisense oligonucleotides reduced CPA-evoked whole-cell currents (Sweeney *et al.* 2002). In addition, in the aorta A7r5 cell line knockdown of endogenous TRPC1 expression with siRNA and antisense methods reduced endogenous whole-cell currents induced by thapsigargin (Brueggemann *et al.* 2006). Moreover Fig. 4*Aa* and *Ba* illustrates work from our laboratory with single channel studies which show that bath application of anti-TRPC1 antibodies, raised against a putative intracellular epitope, to the cytoplasmic surface of inside-out patches produced marked inhibition of SOC activity evoked by CaM in portal vein myocytes (AP Albert, SN Saleh, CM Peppiatt-Wildman & WA Large unpublished data) and of SOCs stimulated by angiotensin II in mesenteric artery (see Saleh *et al.* 2006).

In addition it has been shown that an anti-TRPC5 antibody raised against a putative extracellular epitope inhibited thapsigargin-evoked Ca^{2+} influx in rabbit pial arterioles (Xu *et al.* 2006). Furthermore we have demonstrated that anti-TRPC5 antibodies generated against putative intracellular domains and bath applied to the intracellular surface of inside-out patches from portal vein and mesenteric artery myocytes also reduced SOC activity (Fig. 4*Ab* and *Bb*, AP Albert, SN Saleh, CM Peppiatt-Wildman & WA Large unpublished data). It should be noted that these antibodies display a high degree of selectivity when used in combination with inside-out patch recording of single channel activity since anti-TRPC1 and -TRPC5 antibodies do not inhibit the activity of constitutively active native TRPC3 channels in rabbit ear artery (Albert *et al.* 2006*a*) or receptor-operated TRPC6 (mesenteric artery, Saleh *et al.*

2006) or heterotetrameric TRPC3/C7 channels (coronary artery, Peppiatt-Wildman *et al.* 2007). Moreover TRPC1 and TRPC5 proteins have been shown to co-localize and associate with one another in human saphenous vein (Xu *et al.* 2006). These data suggest that TRPC1/TRPC5 heterotetramers may mediate SOCs in vascular smooth muscle which is supported by the observation that expression of TRPC1/TRPC5 channel proteins produced single channel currents with a unitary conductance of about 5 pS (Strübing *et al.* 2001) which is close to the conductance value of SOCs found in vascular smooth muscle (2–5 pS, Trepakova *et al.* 2001; Golovina *et al.* 2001; Albert & Large, 2002*a*).

These data provide strong evidence that TRPC1, possibly as a heterotetramer with TRPC5, is an essential component of SOCs in vascular smooth muscle. However, it is likely that other TRPC proteins are involved and the difference in biophysical properties between SOCs in aorta myocytes *versus* portal vein/mesenteric myocytes results from different heterotetrameric structures.

Multiple activation mechanisms of TRPCs proposed to mediate SOCs in vascular smooth muscle

We have shown that PKC and CaM can activate SOCs in vascular myocytes and there is support for TRPC1 and TRPC5 being components of these channels. Therefore it is interesting to consider the evidence for activation of expressed TRPC1 and TRPC5 by PKC and CaM.

Stimulation of PKC activates TRPC1. A unique property of TRPC1 is that stimulation of PKC has been shown to be required for its activation by store depletion (Ahmmed *et al.* 2004) whereas the activity of all other TRPCs have been shown to be inhibited by stimulation of this kinase (Soboloff *et al.* 2007). In cultured human umbilical vein endothelial cells pharmacological inhibitors of PKC, expression of a PKCα-defective mutant and an anti-TRPC1 antibody, reduced Ca^{2+} influx and whole-cell currents induced by thrombin, thapsigargin and intracellular application of IP_3. Moreover this study showed that thrombin and thapsigargin produced PKCα-dependent phosphorylation of TRPC1 proteins (Ahmmed *et al.* 2004). These data provide compelling evidence for PKCα-mediated phosphorylation of TRPC1 proteins having a critical role in activating SOC activity. The DAG analogue and PKC activator, OAG, has also been shown to activate expressed TRPC1 (Lintschinger *et al.* 2000). In addition, stimulation of PKCα has also been shown to be essential in the activation of SOCs in mesangial cells (Ma *et al.* 2002) although these channels are proposed to consist of TRPC4 proteins (Wang *et al.* 2004).

It is tempting to speculate that TRPC1 subunits confer PKC sensitivity to SOC activation previously described in portal vein/mesenteric artery myocytes (Fig. 5*Ba*, Albert & Large, 2002*b*; Saleh *et al.* 2006).

Effect of CaM on TRPC1 and TRPC5. It is well established that TRPC proteins contain multiple CaM binding sites

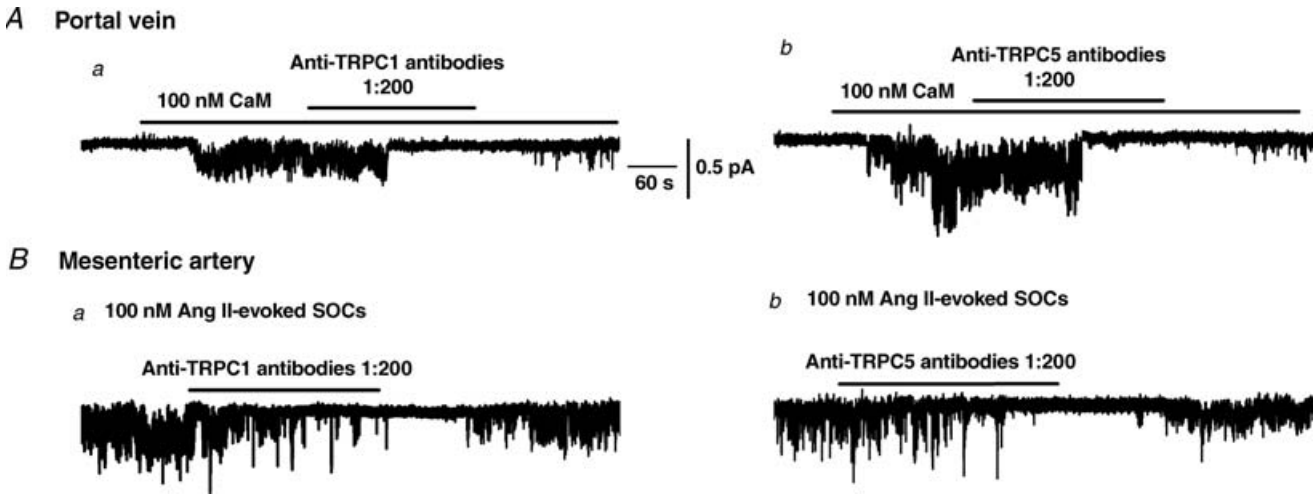

Figure 4. TRPC1 and TRPC5 are important components of SOCs in rabbit portal vein and mesenteric artery myocytes
Aa and *b*, bath application of, respectively, anti-TRPC1 and anti-TRPC5 antibodies reversibly inhibited CaM-evoked SOC activity in inside-out patches from portal vein myocytes at −80 mV. *Ba* and *b*, bath application of, respectively, anti-TRPC1 and anti-TRPC5 antibodies inhibited Ang II-induced stimulation of SOC activity in inside-out patches from mesenteric artery myocytes at −80 mV. *Ba* was reproduced from Saleh *et al.* (2006). *Aa* and *b,* and *Bb* are authors' previously unpublished data.

with all members of the TRPC family containing a conserved region at the carboxyl (C) terminus which binds both CaM and IP_3 receptors (CIRB domain) and some TRPCs also exhibit non-conserved CaM binding sites (Tang *et al.* 2001; see Zhu, 2005 for a detailed review). Functional studies have shown that CaM has mainly an inhibitory action on TRPC channel activity, including TRPC1, by competing with an excitatory effect of IP_3 at the CIRB domain (Zhu, 2005). However, in contrast to these inhibitory actions of CaM on TRPCs, a characteristic feature of TRPC5 channel activity is that it can be facilitated by CaM. Application of intracellular CaM facilitated the peak amplitude and rate of onset of expressed TRPC5-mediated whole-cell currents through a novel non-CIRB binding site, and in addition CaM has also been shown to be important for maintaining agonist-induced TRPC5 activity through the CIRB domain (Ordaz *et al.* 2005). Moreover the Ca^{2+}/CaM-dependent enzyme myosin light chain kinase (MLCK) has also been shown to be essential for initiating activation of TRPC5 channels through a constitutively active trafficking process which regulates transport of TRPC5 channels to the plasma membrane (Kim *et al.* 2006; Shimizu *et al.* 2006).

Therefore these facilitatory actions of CaM on TRPC5 activity may indicate that the excitatory effect of CaM on SOCs in rabbit portal vein is conferred by TRPC5 subunits (see above, Albert *et al.* 2006*a*) and involves a direct interaction with TRPC5 proteins since inhibitors of CaM-dependent enzymes, e.g. MLCK, CaM kinase II and calcineurin, do not inhibit CaM-evoked SOC activity (Albert *et al.* 2006*a*).

In addition, expressed TRPC5 activity has also been shown to be activated by lysophospholipids, including lysophosphatidylinositol (LPI), via a relatively direct

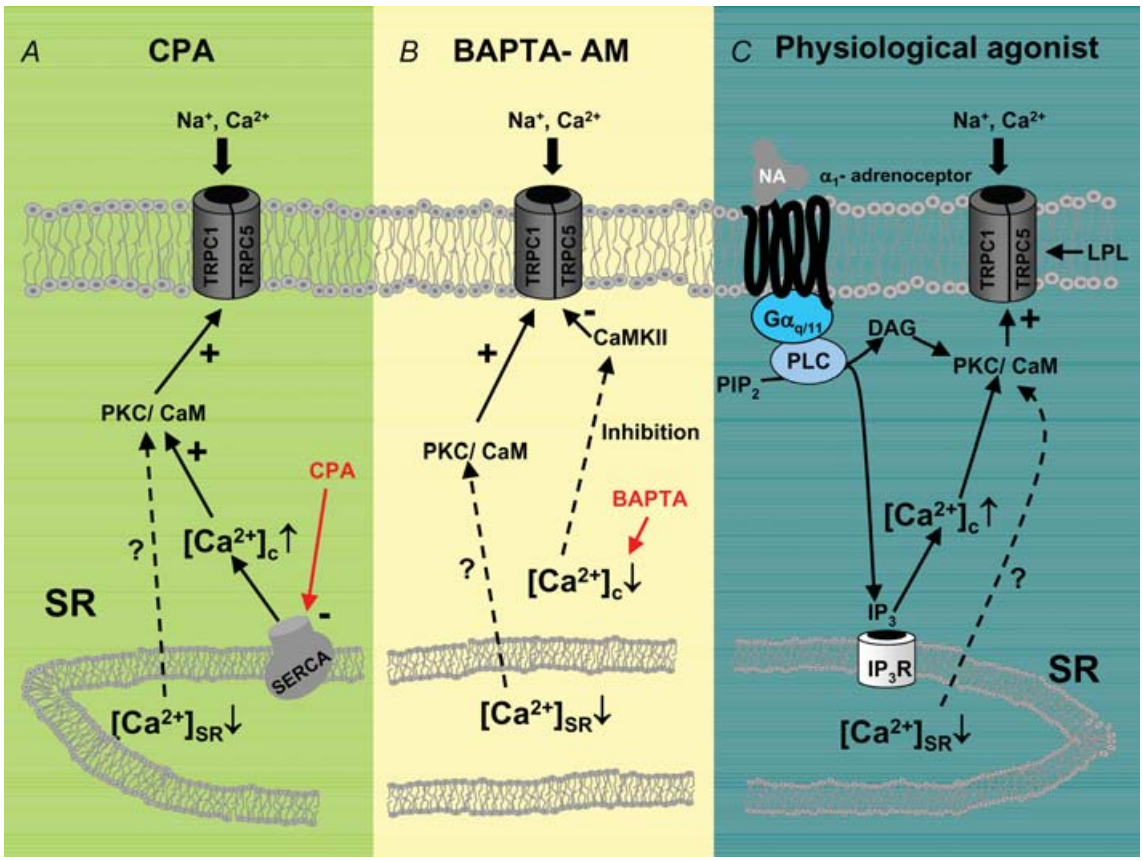

Figure 5. Schematic diagram of multiple activation mechanisms of TRPC1/TRPC5-mediated SOCs in rabbit portal vein myocytes

A, CPA may activate SOCs by depleting Ca^{2+} levels within the SR ($[Ca^{2+}]_{SR}$) leading to stimulation of PKC/CaM by an unknown pathway (dashed lines) which induces channel opening. However, CPA may also induce SOC activity by evoking a rise in cytosolic Ca^{2+} levels ($[Ca^{2+}]_c$) which leads to activation of a Ca^{2+}-sensitive PKC and CaM. *B*, BAPTA-AM may also activate SOCs by depleting $[Ca^{2+}]_{SR}$ but this agent may produce a reduction in $[Ca^{2+}]_c$ to remove the inhibitory action of CaM kinase II (CaMKII) on SOCs leading to stimulation of the channels via the presence of a constitutive driver, e.g. CaM. *C*, noradrenaline (NA) acting at α_1-adrenoceptors activates SOC activity via both store-dependent and -independent pathways involving stimulation of PKC. In addition lysophospholipids (LPLs) may also activate SOC opening.

interaction with the channel proteins (Flemming *et al.* 2005). This may relate to the proposed role of $iPLA_2$-mediated production of LPIs in activating native SOCs in aorta myocytes (Smani *et al.* 2004) and suggest that TRPC5 subunits are also components of these SOCs.

Conclusion

Experimental evidence indicates that it is unlikely that a single mechanism is involved in activating SOCs in smooth muscle and Fig. 5 summarizes our proposal for the activation of SOCs in rabbit portal vein myocytes. We suggest that TRPC1 and TRPC5 proteins are components of SOCs but it is probable that other TRPC subunits and possibly Orai and STIM proteins are involved. Figure 5*A* and *B* shows that CPA and BAPTA-AM may activate SOC activity in portal vein according to the strict definition of SOCs whereby these agents produce a reduction of $[Ca^{2+}]_{SR}$ to activate SOCs. Evidence shows that both CaM and PKC are involved in this process (see earlier) but it is not clear how a reduction in $[Ca^{2+}]_{SR}$ stimulates PKC and CaM. Alternatively it is possible that activation of SOCs by CPA results from an increase of $[Ca^{2+}]_c$ which stimulates Ca^{2+}-sensitive PKC and/or CaM which evoke SOCs (Fig. 5*A*). Also, BAPTA-AM may activate SOCs by reducing $[Ca^{2+}]_c$ to remove the inhibitory effect of CaM kinase II with subsequent opening of channels through constitutive driver activity involving PKC and/or CaM. Finally Fig. 5*C* shows that physiological agonists such as noradrenaline acting on a GPCR linked to the classical phosphoinositol biochemical cascade activates SOCs via two separate pathways: (i) a store-dependent pathway involving production of IP_3 which releases Ca^{2+} from internal stores leading to a rise in $[Ca^{2+}]_c$ and stimulation of PKC/CaM to cause channel opening, and (ii) a store-independent/membrane-delimited pathway involving generation of DAG which stimulates PKC to induce SOC activity. Our evidence suggests that the primary mechanisms of activation of SOCs in smooth muscle in physiological conditions involve PKC and CaM mechanisms, perhaps mediated by changes in $[Ca^{2+}]_c$. In addition it is possible that several cellular mechanisms contribute to the whole-cell current or intracellular Ca^{2+} signal produced by agents that deplete intracellular Ca^{2+} stores.

In summary there is strong evidence that vascular smooth muscle expresses diverse SOCs that differ in biophysical properties and activation mechanisms. This heterogeneity in channel properties is likely to be mediated by TRPC proteins assembled together into different heterotetrameric channel structures.

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Note added in proof

Recently Dietrich *et al.* (2007) have demonstrated that whole-cell currents induced by CPA and IP_3 are similar in vascular myocytes from wild-type and $TRPC1^{-/-}$ mice. Thus it appears that in these experiments TRPC1 subunits do not contribute to the SOC signal in contrast to the CPA-evoked and PKC-mediated single channel currents described in the present review which do seem to involve TRPC1 proteins. These data further support our hypothesis that diverse molecular components and activation mechanisms contribute to cellular signals that are activated by agents that deplete intracellular Ca^{2+} stores.

Acknowledgements

The work in the laboratory of the authors was supported by The Wellcome Trust and The British Heart Foundation.