SYMPOSIUM REPORT

Signal transduction pathways and gating mechanisms of native TRP-like cation channels in vascular myocytes

A. P. Albert and W. A. Large

Ion channels and Cell Signalling, Division of Basic Medical Sciences, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK

Activation of Ca^{2+}-permeable non-selective cation channels produces an increase in excitability of vascular smooth muscle cells which has an important role in vasoconstriction. These channels are activated by various physiological stimuli including vasoconstrictor agents such as noradrenaline, depletion of internal Ca^{2+} stores and cell stretching. In addition cation channels have been shown to be constitutively active and these channels are thought to contribute to resting membrane conductance and basal Ca^{2+} influx in vascular myocytes. Recent evidence has suggested that transient receptor potential (TRP) proteins represent strong candidates for these channels in the vasculature. This review discusses proposed signal transduction pathways and gating mechanisms which link physiological stimuli to opening of cation channels in vascular myocytes. It is apparent that G-protein-coupled pathways linked to stimulation of phospholipase activity have a profound effect on regulating channel activity and that generation of diacylglycerol (DAG) is a central event in these signalling cascades with this triglyceride having a pivotal role in gating cation channels via both PKC-*independent* and -*dependent* mechanisms. Moreover phosphorylation processes produced by stimulation of protein kinases have been proposed to have an important role in regulating cation channel activity.

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Corresponding author A. P. Albert: Ion channels and Cell Signalling, Division of Basic Medical Sciences, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK. Email: aalbert@sgul.ac.uk

Introduction

It is well established that plasmalemmal ion channels have a central function in regulating contractility of vascular smooth muscle cells and recently much attention has focused on Ca^{2+}-permeable non-selective cation channels. Activation of these channels produces depolarization leading to opening of voltage-dependent calcium channels (VDCCs) and subsequent vasoconstriction and also provides Ca^{2+} entry generating vasoconstriction independently of VDCCs (Large, 2002). There is now much evidence suggesting that transient receptor potential (TRP) proteins are strong candidates for the molecular identity of cation channels in the vasculature (Xu & Beech, 2001; Inoue *et al.* 2001; Large, 2002; Sweeney *et al.* 2002; Welsh *et al.* 2002; Muraki *et al.* 2003; Beech *et al.* 2004; Earley *et al.* 2004).

This review discusses signal transduction pathways and gating mechanisms regulating TRP-like cation channels in vascular myocytes (for details of biophysical properties see Large, 2002; Albert & Large, 2003*a*; Beech *et al.* 2004). We have classified the cation channels into four major groups according to the primary physiological stimuli. First, there are receptor-operated channels (ROCs), which are activated by excitatory agents such as noradrenaline to produce vasoconstriction (Large, 2002). Secondly, there are constitutively active cation channels (CCCs), which contribute to the resting membrane conductance and basal Ca^{2+} influx (Bae *et al.* 1999; Terasawa *et al.* 2002; Albert *et al.* 2003; Thorneloe & Nelson, 2004; Petkov *et al.* 2005). Thirdly, store-operated channels (SOCs), which are activated in response to depletion of internal Ca^{2+} stores and also contribute to vasoconstriction and cell proliferation (McFadzean & Gibson, 2002; Sweeney *et al.* 2002; Albert & Large, 2003*a*). Finally, stretch-activated channels (SACs), which contribute to myogenic vasoconstriction (Schubert & Mulvany, 1999).

With all of the above groups of ion channels it is likely that well-established G-protein-coupled biochemical cascade systems are important in regulating cation channel activity. Moreover it appears that production of diacylglycerol (DAG) is pivotal to the activation of many

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of these channels but that pathways leading to generation of DAG and mechanisms by which DAG opens channels vary for different classes of ion channels and vascular preparations.

Receptor-operated cation channels (ROCs)

The first ROC to be described in vascular smooth muscle was a noradrenaline-evoked cation conductance in rabbit portal vein myocytes (Byrne & Large, 1988) and strong evidence indicates that this conductance is mediated by a TRPC6-like channel protein (Inoue *et al.* 2001). Figure 1 shows that activation of these channels involves the classical G-protein-coupled phosphoinositol system (PI) involving stimulation of PI-phospholipase C (PI-PLC) and production of DAG (Helliwell & Large, 1997). A surprising and novel result from the study of Helliwell & Large (1997) was that generation of endogenous DAG induced by the DAG lipase inhibitor RHC80267 and the DAG analogue 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) activated ROCs via a protein kinase C (PKC)-*independent* mechanism (Fig. 1). Gating by DAG of several non-selective cation channels has been subsequently described including TRPC3, 6 and 7 channel proteins expressed in cell lines (e.g. Hofmann *et al.* 1999; Inoue *et al.* 2001; Estacion *et al.* 2004; Shi *et al.* 2004) and it has been shown that TRPC6 and TRPC3 proteins are components of native ROCs in portal vein and cerebral artery myocytes (Inoue *et al.* 2001; Reading *et al.* 2005).

A key observation in rabbit portal vein was that whole-cell responses to OAG were considerably slower in onset and smaller in amplitude than responses to noradrenaline (Helliwell & Large, 1997; Albert & Large, 2003*b*). This was puzzling and suggested that DAG may not be the only molecule activating the channel. This conundrum was resolved by the demonstration of a novel synergistic effect of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) on OAG-activated ROCs in rabbit portal vein myocytes where $Ins(1, 4,5)P_3$ potentiated OAG-evoked responses although $Ins(1,4,5)P_3$ had no effect on its own (Fig. 1; Albert & Large, 2003*b*). The mechanism underlying this synergism was heparin insensitive indicating that classical $Ins(1,4,5)P_3$ receptors on internal Ca^{2+} stores were not involved and suggesting a novel action of $Ins(1,4,5)P_3$ on channel proteins. Co-application of OAG and $Ins(1,4,5)P_3$ mimicked noradrenaline-evoked responses suggesting that both these second messengers are required to produce optimal channel activation and indicating a significant physiological role for these agents in gating ROCs (Albert & Large, 2003*b*).

It has also been shown that a TRPC6-like conductance is stimulated by vasopressin, 5-hydroxytryptamine and platelet-derived growth factor in an aortic A7r5 cell line (Jung *et al.* 2002) indicating that activation of this conductance may be a common vasoconstrictor mechanism.

Stimulation of non-G-protein-coupled receptors has also been shown to activate ROCs in vascular myocytes and much of this work has concentrated on the role of tyrosine kinases, which are proposed to be involved in regulating vasoconstriction and cell proliferation (Hollenberg, 1994). Figure 1 shows that stimulation of tyrosine kinase receptors (TKRs) activate ROCs and although the pathways linking TKRs to opening of ROCs is unknown it is possible that stimulation of TKRs activates PLCγ leading to production of DAG and channel opening

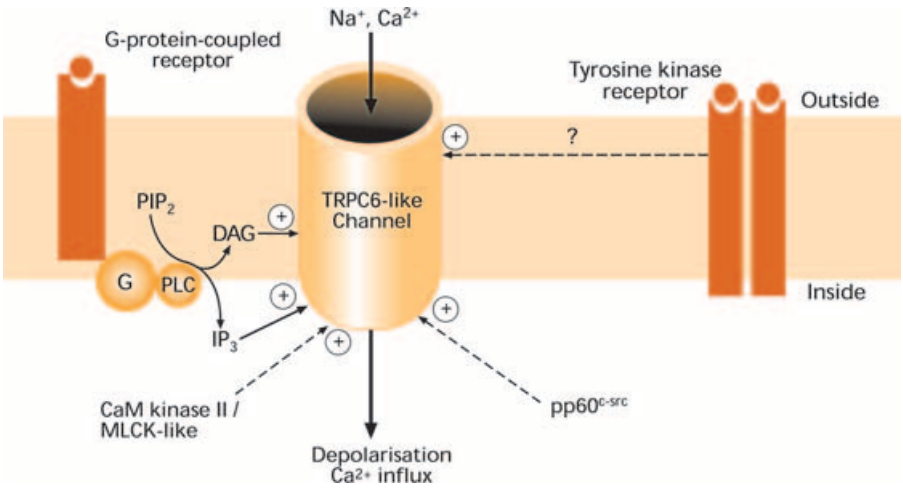

Figure 1. Regulation of ROCs by signal transduction pathways in rabbit portal vein myocytes
Stimulation of G-protein-coupled receptors activates ROCs through the classical PI system, which generates DAG leading to channel opening via a PKC-*independent* mechanism. Activation of tyrosine kinase receptors also activates channel opening. Note that $Ins(1,4,5)P_3$, $pp60^{c\text{-}src}$ and a phosphorylation step involving a CaM kinase II/MLCK-like molecule have all been shown to be involved in activating ROCs.

(Albert *et al.* 2001; Jung *et al.* 2002). An interesting result is that cytosolic tyrosine kinases (CTKs), which are stimulated by both G-protein-coupled and TKR pathways, also activate ROCs (Albert *et al.* 2001; Hollenberg, 1994). In rabbit portal myocytes pp60$^{c\text{-}src}$, a member of the src family of CTKs, has been shown to activate TRPC6-like activity (Albert *et al.* 2001) although the mechanism of pp60$^{c\text{-}src}$ on channel activity is also not understood. However, an N-terminal region of TRPC6 proteins interacts with and is phosphorylated by Fyn, another member of the src family of CTKs, and Fyn was shown to increase OAG-evoked TRPC6 channel activity (Hisatsune *et al.* 2004).

Constitutive cation channels (CCCs)

The resting membrane potential of most smooth muscle preparations is substantially more positive than the potassium equilibrium potential indicating the presence of a spontaneous depolarizing mechanism(s). In rabbit ear artery CCCs have been described which have similar, although not identical, biophysical properties to TRPC6-like channels in portal vein (Albert & Large, 2001; Albert *et al.* 2003). However, the regulation of CCCs in ear artery is complex compared to ROCs in portal vein in that OAG produces dual effects of both excitation and inhibition on channel activity via parallel signal transduction pathways, and these pathways are summarized in Fig. 2 (Albert & Large, 2004; Albert *et al.* 2005). In this preparation it appears that PI-PLC is primarily involved in an inhibitory pathway unlike ROCs in portal vein where this phospholipase is primarily involved in excitation (Helliwell & Large, 1997). Moreover it appears that constitutively active $G_{\alpha q/\alpha 11}$ stimulates PI-PLC to produce DAG, which activates PKC to inhibit channel activity (Fig. 2; Albert & Large, 2004). The excitatory pathway is driven by constitutive $G_{\alpha i/\alpha o}$ stimulation of phosphatidylcholine-phospholipase D (PC-PLD), which generates, via phosphatidic acid, DAG to excite channel activity (Fig. 2; Albert & Large, 2004; Albert *et al.* 2005).

It should be noted that noradrenaline has been shown to enhance the activity of CCCs in ear artery indicating that these channels may also be implicated in vasoconstrictor-mediated responses (Albert & Large, 2004) although we believe the constitutive property to be of primary importance.

The biophysical properties and activation by DAG via a PKC-*independent* mechanism of CCCs in rabbit ear artery are similar, but not identical, to the characteristics of TRPC6-like channels in portal vein. This suggests that CCCs belongs to the TRPC3,6,7 subgroup of channel proteins. Moreover in expression systems there is evidence that TRPC3 and TRPC7 display constitutive activity (Zitt *et al.* 1997; Okada *et al.* 1999) and since TRPC7 does not appear to be present in rabbit ear artery myocytes (authors' unpublished data) it is possible that TRPC3 may have an important role in CCCs in this preparation.

Store-operated channels (SOCs)

Depletion of internal Ca^{2+} stores activates plasmalemmal Ca^{2+}-permeable channels, termed SOCs, which are thought to provide an influx of Ca^{2+} involved in replenishing Ca^{2+} stores, vasoconstriction and cell proliferation (McFadzean & Gibson, 2002; Sweeney *et al.* 2002; Albert & Large, 2003*a*). It has been shown that depletion of internal Ca^{2+} stores by the SR Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid (CPA) activates at least two different types of SOCs in vascular smooth muscle, which have similar unitary conductance values (2–3 pS; Trepakova *et al.* 2001; Albert & Large, 2002*a*) but are distinguished from one another by different permeabilities to Ca^{2+} ions and activation mechanisms, and these activation pathways are shown in Fig. 3.

In portal vein myocytes SOCs which have a high permeability to Ca^{2+} ions ($P_{Ca}/P_{Na} \approx 50$) are activated by OAG but in contrast to ROCs in portal vein this triglyceride

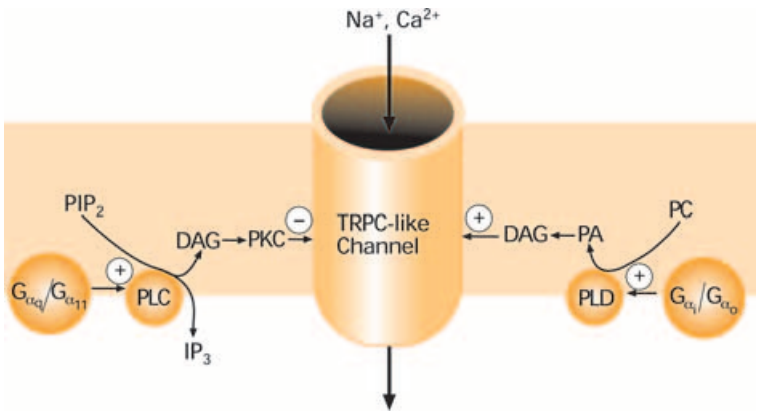

Figure 2. Activity of CCCs is regulated by parallel signal transduction pathways in rabbit ear artery myocytes
Spontaneously active $G_{\alpha i/o}$ proteins activate CCCs by stimulating PC-PLD, leading to hydrolysis of PC and generation of DAG via phosphatidic acid, which opens channels by a PKC-independent mechanism. In parallel to this excitatory pathway spontaneously active $G_{\alpha q/\alpha 11}$ proteins inhibit CCC activity through a PKC-dependent mechanism involving stimulation of the classical PI biochemical cascade system.

opens SOCs via a PKC-*dependent* mechanism (Fig. 3, Albert & Large, 2002*a*,*b*; Liu *et al.* 2005*a*,*b*). It is not known how depletion of internal Ca^{2+} stores is linked to activation of PKC and opening of SOCs but phosphorylation of the channel protein has been proposed (Liu *et al.* 2005*a*,*b*).

Interestingly SOC activity is potentiated by Ins(1,4,5)P_3 via a similar heparin-sensitive mechanism to the action of Ins(1,4,5)P_3 on OAG-activated ROCs, suggesting that ROCs and SOCs in portal vein possess some similar features of the activation mechanism (see above; Albert & Large, 2003*b*; Liu *et al.* 2005*b*).

Figure 3 shows that another SOC described in aortic myocytes has a relatively low permeability to Ca^{2+} ions ($P_{Ca}/P_{Na} \approx 1$) and is activated by lysophospholipids (LPLs), which are products of the action of phospholipase A_2 (PLA_2) on PC (Smani *et al.* 2004). This model involves the release of a calcium influx factor (CIF) from depleted internal Ca^{2+} stores, which displaces calmodulin (CaM) from membrane-bound Ca^{2+}-independent PLA_2 resulting in activation of PLA_2 and generation of LPLs (Smani *et al.* 2004). However, the identity of CIF is unknown, which precludes a clear understanding of this mechanism.

With regard to the molecular identity of SOCs, there are many studies in expression systems which have shown that several members of the TRP family can behave as SOCs (Parekh & Putney, 2005). In vascular smooth muscle only TRPC1 has been implicated as a component of SOCs (Xu & Beech, 2001; Sweeney *et al.* 2002) and interestingly it has been shown that activation of TRPC1 proteins by store depletion required PKC-mediated phosphorylation of the channel protein (Ahmmed *et al.* 2004).

Stretch-activated cation channels (SACs)

It is well known that increased intralumenal pressure in small arteries and arterioles activates vasoconstriction, the myogenic response, which has an important role in controlling tissue blood flow (Schubert & Mulvany, 1999). The myogenic response is stimulated by changes in tension of vascular myocytes producing membrane depolarization and subsequent rise in intracellular Ca^{2+} concentration due to influx of Ca^{2+} through opening of VDCCs (Schubert & Mulvany, 1999). Different ion channels have been proposed to initiate the depolarizing phase of the myogenic response including SACs, which have been described in a number of different vascular preparations (Setoguchi *et al.* 1997; Schubert & Mulvany, 1999; Welsh *et al.* 2000; Wu & Davis, 2001; Muraki *et al.* 2003; Park *et al.* 2003). Like many cation channels in vascular myocytes, the transduction pathways linking physiological stimuli (e.g. changes in tension) to membrane depolarization are poorly understood, but similar to the cation channels described above, DAG production may play a central role.

In rat cerebral artery myocytes, PKC activators increased SAC activity and induced depolarizations, which were blocked by a PKC inhibitor (Slish *et al.* 2002). In rabbit pulmonary artery myocytes, SACs were

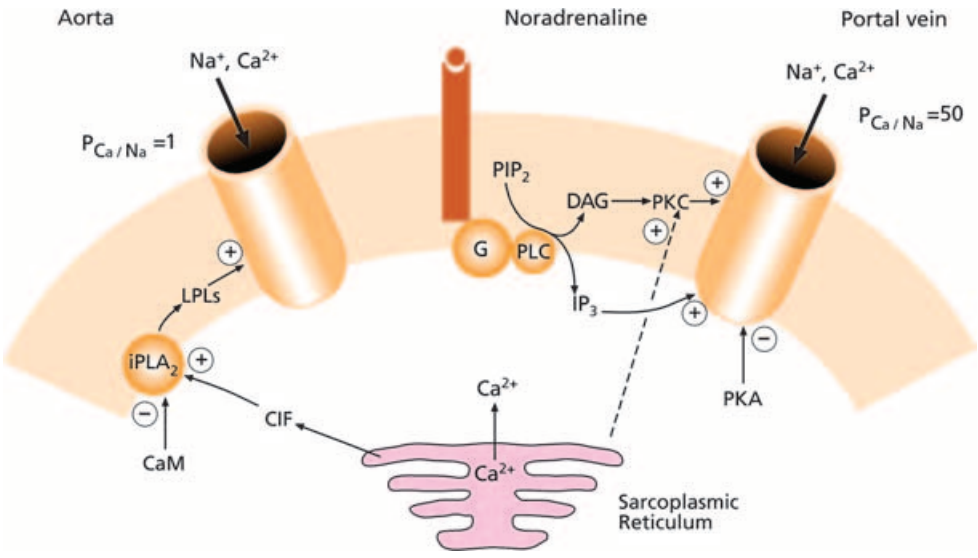

Figure 3. Activation mechanisms of SOCs in vascular myocytes
The activation mechanisms of SOCs in aortic and portal vein myocytes are shown. In aortic myocytes SOCs are activated by LPLs produced by stimulation of $iPLA_2$ with this phospholipase regulated by release of CIF from depleted internal Ca^{2+} stores. In portal vein SOCs are activated by a store-dependent process involving activation of PKC via an unknown pathway (denoted by dashed line) and by a store-independent pathway involving G-protein-coupled receptors which are also linked to activation of PKC.

blocked by inhibitors of PI-PLC and increased by a DAG analogue, and moreover in rabbit basilar arteries inhibition of PI-PLC reduced the myogenic response produced by tissue stretching (Park *et al.* 2003). These studies suggest that increases in tension of vascular myocytes lead to stimulation of PI-PLC and production of DAG, which activates PKC producing a myogenic response. In rat cerebral artery TRPC6 antisense treatment reduced SAC activity, pressure-induced depolarization and myogenic tone suggesting that these proteins are important components of SACs (Welsh *et al.* 2002). However a difficulty is that TRPC6 channel proteins have previously been shown to be opened by DAG via a PKC-*independent* mechanism (see earlier discussion on ROCs) and therefore it seems likely that other ion channels/biochemical pathways which are regulated by PKC are also involved. Recent studies have proposed that TRPM4 channel proteins are also involved in activating the myogenic response in rat cerebral arteries but the link between TRPC6 and TRPM4 is uncertain (Earley *et al.* 2004). In addition TRPV2 proteins are thought to compose osmotically sensitive cation channels in mouse aortic myocytes (Muraki *et al.* 2003), and TRPC1 proteins have been shown to compose a mechanosensitive cation channel in frog oocytes (Maroto *et al.* 2005).

Gating mechanisms of cation channels in vascular myocytes

Does DAG directly activate non-selective cation channels in vascular smooth muscle? It is evident that in many vascular preparations DAG has a pivotal role in gating some cation channels (e.g. ROCs) via a PKC-*independent* mechanism but it is not understood how DAG opens the channel. It is likely that DAG either directly gates channels or a metabolite of DAG opens these channels. To address this problem studies have used well-characterized inhibitors of DAG metabolism pathways such as the DAG lipase inhibitor RHC80267 and the DAG kinase inhibitor R54459, which prevent metabolism of DAG into arachidonic acid products and phosphatidic acid, respectively, to increase endogenous levels of DAG. These data show that RHC80267 activates ROCs in portal vein (Helliwell & Large, 1997) and both RHC80267 and R54459 enhance CCC activity in ear artery (Albert *et al.* 2005), which suggests that increased production of endogenous DAG and not formation of DAG metabolites is involved in activating these channels. Activation of expressed TRPC6 isoforms, which are essential components of ROCs in portal vein (Inoue *et al.* 2001), have shown that an extended N-terminus region of rat TRPC6A is crucial for channel activation by DAG (Zhang & Saffen, 2001). In conclusion these data indicate that DAG is the signal molecule so far identified that is closest to channel gating.

Role of phosphorylation. Irrespective of the potential direct actions of DAG on channel gating there is evidence which suggests that phosphorylation may also have an important role in activating cation channels in vascular myocytes. In portal vein a phosphorylation process has been shown to be important for activation of TRPC6-like channels by noradrenaline and DAG and this involved a Ca^{2+}/CaM-dependent kinase, possibly a myosin light chain (MLCK)/MLCK-like molecule (Fig. 1; Aromolaran *et al.* 2000). Moreover activation of TRPC6 proteins expressed in HEK 293 cells by receptor agonists, G-protein activators or DAG also required a phosphorylation process, but in contrast to portal vein these processes involved CaM kinase II (Shi *et al.* 2004). These data indicate that activation of ROCs may have a complex gating mechanism which requires a priming phosphorylation step before opening by DAG via a PKC-*independent* mechanism.

In portal vein DAG activates SOCs via the conventional pathway by stimulating PKC activity, which leads to channel opening, presumably by phosphorylating serine/theonine residues within channel proteins. Expressed TRPC1 proteins have been proposed as candidates for the molecular identity of SOCs in vascular myocytes (Xu & Beech, 2000) and these proteins have been shown to be activated by store depletion via a PKC-*dependent* mechanism which involves direct phosphorylation of the TRPC1 channel protein (Ahmmed *et al.* 2004).

Data also indicate that phosphorylation may be involved in inhibiting SOCs and ROCs in vascular myocytes. In portal vein stimulation of β-adrenoceptors which are involved in vasodilatation reduced SOC activity via a cAMP-dependent kinase mechanism (Fig. 3; Liu *et al.* 2005*a*) and expressed TRPC6 proteins were also inhibited by PKC in a Ca^{2+}-dependent manner (Shi *et al.* 2004).

Multi-modal gating of native non-selective cation channels in vascular smooth muscle. There is increasing evidence suggesting that the same cation channels in vascular myocytes can be activated by different physiological signals, which is termed multi-modal gating. An important discovery showed that SOCs in portal vein, recorded in outside-out patches that did not respond to CPA and therefore did not contain functional internal Ca^{2+} stores, could be activated by noradrenaline via a store-independent pathway (e.g. they can behave as ROCs; Albert & Large, 2002*b*). The biochemical basis for different physiological signals converging on the same channel may be due to involvement of similar signal transductions pathways and this appears to be the case with SOCs in portal vein where activation of PKC is involved in channel opening by both store depletion and receptor stimulation (Fig. 3; Albert & Large, 2002*b*). Alternatively physiological stimuli may be linked to opening of the same cation channel via parallel biochemical cascades which act

upon the same channel protein. This may be apparent with the TRPC6-like conductance in portal vein, which can be activated by stimulation of G-protein-coupled receptors, RTKs and CTKs (Helliwell & Large 1997; Albert *et al.* 2001). Moreover a TRPC6-like conductance is suggested to be activated by cell stretching in cerebral arteries suggesting that these channels are also mechano-sensitive (Welsh *et al.* 2002). It is also interesting to note that ROCs and SOCs in portal vein can display constitutive activity (Albert & Large, 2001, 2002*a*) and noradrenaline acting at G-protein-coupled receptors enhances activity of CCCs in ear artery.

These data indicate that characterizing cation channels in vascular smooth muscle into groups based upon activation by physiological stimuli is not completely satisfactory and that these channels should be considered promiscuous with many physiological stimuli linked to channel gating.

Conclusion

In conclusion there are several classes of Ca^{2+}-permeable cation channels in vascular smooth muscle and there is growing evidence that TRP proteins form these channels. Moreover these channels are controlled by classical transduction pathways with DAG having an pivotal role in regulating channel activity via PKC-independent and -dependent actions and that different signalling cascades are involved in the production of DAG.

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