

4. Discussion

The major finding of the present study is that TRPC channels play a role in AVP-induced nonselective cation currents in A7r5 smooth muscle cells. More specifically, in light of the combined Ca^{2+} imaging, electrophysiological, and molecular biological data, TRPC6 is suggested to be a property-determining molecular component of the channels mediating these currents.

To evaluate the role of TRPC channels in A7r5 smooth muscle cells, the properties of the nonselective cation currents endogenous to this cell type were compared with the properties reported for currents mediated by heterologously-expressed TRPC channel proteins. Electrophysiological data in support of an involvement of the TRPC3/6/7 group in receptor-stimulated cation currents in A7r5 smooth muscle cells is summarized in Table 3 and discussed below. As mentioned in the introduction, there is no general consensus on the properties of recombinant TRPC channel isoforms. For reasons of clarity, these discrepancies are not incorporated in Table 3. Here, only results obtained by our laboratory and confirmed by independent research groups (or, for TRPC5 and TRPC6, by results of the present study) are considered, but discrepant reports are addressed in the text. TRPC2 is not included in the table because no biophysical data are available for recombinant TRPC2. Moreover, its physiological role is thought to be confined to specific functions in specialized tissues, such as the vomeronasal organ and sperm (Hofmann *et al.*, 2000; Jungnickel *et al.*, 2001).

Doubly-rectifying I-V relation

In the present study, AVP was found to induce nonselective cation currents in A7r5 cells. These currents displayed a characteristic doubly-rectifying *I-V* relationship that strongly resembles the *I-V* relationship described for recombinantly expressed hTRPC3/6 (Hofmann *et al.*, 2000; Kiselyov *et al.*, 2000), mTRPC7 (Okada *et al.*, 1999) and mTRPC4/5 (Schaefer *et al.*, 2000; Schaefer *et al.*, 2002). Similar *I-V* relations for TRPC5 have also been presented by other groups (Okada *et al.*, 1998; Strübing *et al.*, 2001). However, in the latter studies, the *I-V* relationships are only shown for voltages ranging from -100 to +50 mV and the current is suggested to be exclusively inwardly-

rectifying. By contrast, linear or I_{CRAC} -like (inwardly-rectifying, with reversal potential positive to +50 mV in physiological solutions, cf. Hoth & Penner, 1992) $I-V$ relations have been reported for TRPC4 by McKay *et al.* (2000) and Warnat *et al.* (1999), respectively. For recombinant TRPC1, only linear $I-V$ relations have been reported (Zitt *et al.*, 1996; Sinkins *et al.*, 1998; Lintschinger *et al.*, 2000) and no data are available for TRPC2. Importantly, with the exception of TRPC4/5 and TRPC3/6/7, to date, no other ion channel proteins are known that pass currents with a similar doubly-rectifying $I-V$ relation.

Table 3: Comparison of receptor-activated cation currents endogenous to A7r5 smooth muscle cells with currents mediated by heterologously-expressed TRPC channel proteins

Current properties	A7r5	TRPC1	TRPC3,6,7	TRPC4, 5
Doubly-rectifying $I-V$ relation	✓	✗	✓	✓
PLC-dependent, but store-depletion independent activation	✓	✗ ¹	✓	✓
Activation by DAG analogues	✓	?	✓	✗
Current inhibition by micromolar La^{3+}	✓	✓	✓	✗ ²
Current increase in reduced $[Ca^{2+}]_o$	✓	✓	✓	✗ ³

¹ store-operated activation
² current increased by micromolar La^{3+}
³ current decreased by reduced $[Ca^{2+}]_o$

The activation of nonselective cation currents by AVP in A7r5 smooth muscle cells has been described in a number of previous studies (Van Renterghem *et al.*, 1988; Krautwurst *et al.*, 1994; Nakajima *et al.*, 1996; Iwasawa *et al.*, 1997; Iwamuro *et al.*, 1998). However, none of these studies was intended to clarify the molecular identity of the channels that mediated the observed currents. Surprisingly, in all but one early study by Nakajima *et al.* (1996), receptor-stimulated cation currents in A7r5 cells were shown to display linear $I-V$ relationships. In the study by Nakajima *et al.* (1996), the reported $I-V$ relation of the AVP-induced current was very similar to that described in the present

study. Strangely, all subsequent publications from the same research group (Asano *et al.*, 1997; Iwasawa *et al.*, 1997; Nakajima *et al.*, 1997; Asano *et al.*, 1998) exclusively show linear I - V relationships for AVP-induced currents in A7r5 cells, although the recording conditions were identical. Under the conditions used in the present study, AVP-induced currents with linear I - V relations were never observed. The reasons for the discrepant findings are completely unclear. Interestingly, other properties of the current or Ca^{2+} entry pathway, including PLC-dependent but store depletion-independent activation (Krautwurst *et al.*, 1994; Nakajima *et al.*, 1996), single-channel conductance (Nakajima *et al.*, 1996), inhibition by di- and trivalent cations and SKF 96365 (Krautwurst *et al.*, 1994; Nakajima *et al.*, 1996; Iwamuro *et al.*, 1998), and negative feedback of PKC activation (Kaplan-Albuquerque & Di Salvo, 1998; Broad *et al.*, 1999), are essentially consistent with the findings presented here.

PLC-dependent, but store-depletion independent mode of activation

In the present study, activation of the cation current endogenous to A7r5 cells was shown to be dependent on PLC activation. However, neither InsP_3 nor elevated $[\text{Ca}^{2+}]_i$ directly activated the current. Likewise, store-depletion protocols failed to activate the current. Current activation was also unaffected by infusion of heparin, which rules out an involvement of InsP_3 receptors in channel activation. Comparable PLC-dependent but store depletion-independent activation mechanisms have been described for mTRPC4/5 and hTRPC4 (Okada *et al.*, 1998; McKay *et al.*, 2000; Schaefer *et al.*, 2000; Strübing *et al.*, 2001; Schaefer *et al.*, 2002), hTRPC3/6 (Hofmann *et al.*, 1999; Zhang & Saffen, 2001; Delmas *et al.*, 2002) and mTRPC7 (Okada *et al.*, 1999). To complete the picture, it has to be added that while most studies report that TRPC3/6/7 are activated independently of store depletion (for review see Zitt *et al.*, 2002) the situation is less clear for TRPC4/5. According to Flockerzi and colleagues (Philipp *et al.*, 1998; Warnat *et al.*, 1999) and two other studies on TRPC4-expressing *Xenopus* oocytes (Tomita *et al.*, 1998; Kinoshita *et al.*, 2000), TRPC4 and TRPC5 are thought to form store-operated channels. Accordingly, small store-operated currents observed in mouse aortic endothelial cells (MAECs) prepared from wild type mice were found to be almost completely absent in MAECs from mice deficient in TRPC4 (Freichel *et al.*, 2001). Importantly, however, in the above study, no attempts were made to evaluate the effects

of the disruption of the *trpc4* gene on receptor-stimulated currents activated independently of store-depletion.

With the exception of one study (Lintschinger *et al.*, 2000), TRPC1 has been reported to be activated by InsP₃ infusion and store depletion (Zitt *et al.*, 1996; Liu *et al.*, 2000; Lockwich *et al.*, 2000; Singh *et al.*, 2000; Rosado & Sage, 2001; Singh *et al.*, 2001; Delmas *et al.*, 2002). The activation mechanism for recombinant TRPC2 is unclear. Liman *et al.* (1999) suggest store-depletion independent activation for TRPC2, but do not provide any functional data. By contrast, Vannier *et al.* (1999) reported that recombinant mTRPC2 forms store-operated channels. According to a third study (Hofmann *et al.*, 2000), TRPC2 is not involved in either store-operated or store depletion-independent Ca²⁺ entry.

Interestingly, previous studies (Byron & Taylor, 1995; Broad *et al.*, 1999) suggest that Ca²⁺ entry into A7r5 cells, in addition to a non-capacitative component, also comprises a capacitative component. In fluorometric experiments, the two components were found to differ in their permeability to Sr²⁺ and Mn²⁺ and in their sensitivity to block by Gd³⁺ (Broad *et al.*, 1999). By contrast, Ca²⁺ imaging experiments performed in the present study did not reveal a prominent capacitative Ca²⁺ entry component either in response to AVP, or to other protocols known to deplete internal Ca²⁺ stores. Likewise, in whole-cell experiments, no additional current component was detected in response to store depletion or application of AVP, which is in agreement with a study by Iwasawa *et al.* (1997). Thus, a store depletion-activated nonselective cation channel does not exist in A7r5 cells. Since experiments were not performed in raised [Ca²⁺]_o, conditions normally used to measure I_{CRAC}, the presence of a highly Ca²⁺-selective current of this type cannot be completely excluded. However, from the imaging data, such a current is unlikely. The AVP-induced current recorded in the present study shares some properties with the store depletion-independent Ca²⁺ entry component in A7r5 smooth muscle cells identified by Broad *et al.* (1999). Both are insensitive to store depletion and have similar sensitivities to Gd³⁺, being inhibited by 100 μM Gd³⁺, but not by 1 μM Gd³⁺. The latter concentration was shown to completely and irreversibly inhibit the capacitative component (Broad *et al.*, 1999). In our hands, the native cation current was also activated by OAG (see below), but not by arachidonic acid. This contrasts with the

results of Broad *et al.* (1999) who showed that the non-capacitative Ca^{2+} entry pathway was activated by arachidonic acid. The reason for the contradiction between results obtained in the present study and those in studies by Taylor and colleagues (Byron & Taylor, 1995; Broad *et al.*, 1999), especially with respect to the different Ca^{2+} entry pathways in A7r5 cells and their respective properties, are unclear. A7r5 cells obtained from Dr. Colin Taylor, were found to be indistinguishable in their $[\text{Ca}^{2+}]_i$ and current responses to those used in our laboratory. Hence, differences in the A7r5 subclones or the recording techniques used are not valid explanations for the observed discrepancies.

Although in the present study, it was not possible to demonstrate directly that the AVP-stimulated cation channels in A7r5 cells are permeable to Ca^{2+} , owing to the inhibition of cation currents by Ca^{2+} and other divalent cations, similarities in the properties of the AVP-induced Ca^{2+} signal and the cation currents suggest that they are mediated by the same channel. Both responses are transient and have similar time-courses. Furthermore, inhibitory effects of Mn^{2+} were observed for AVP-induced cation currents as well as for the Ca^{2+} influx component in Ca^{2+} imaging experiments. Accordingly, a recent report by Moneer & Taylor (2002) suggests that only the store-depletion independent Ca^{2+} entry pathway is functional during receptor-stimulation.

Current activation by DAG analogues

An important feature of the cation current endogenous to A7r5 cells, revealed in the present study, is its activation by the membrane-permeable DAG analogue OAG. The effects of OAG on channel activation in A7r5 cells seem to be direct and not mediated by subsequent activation of PKC. Activation of the current by OAG supports the involvement of TRPC channels and, more importantly, provides a pointer to the TRPC isoforms that may be involved. This is because PKC-independent current activation by application of DAG analogues is a characteristic feature of the TRPC3/6/7 group of TRPC channels (Hofmann *et al.*, 1999; Okada *et al.*, 1999; Inoue *et al.*, 2001; Zhang & Saffen, 2001; Delmas *et al.*, 2002); also compare results on rTRPC6 in the present study), not shared with the TRPC4/5 group (Hofmann *et al.*, 1999; Schaefer *et al.*, 2000; Strübing *et al.*, 2001; Schaefer *et al.*, 2002), or with TRPC1 (Delmas *et al.*, 2002). The

situation is not entirely clear for TRPC1 because Lintschinger *et al.* (2000) reported activation by OAG for TRPC1.

The fact that the properties of the OAG-induced current in A7r5 cells are virtually identical to those of the AVP-induced current and that in the majority of cells tested, AVP- and OAG-induced currents were not additive, supports the notion that both currents are indeed mediated by the same channels. Considering that both substances exert their effect at different sites of the signal transduction cascade leading to current activation (OAG presumably much more distally than AVP), it is not surprising that small additive current increases were seen in some experiments. Furthermore, the fact that only OAG applied after AVP but not vice versa could in rare cases ($n = 2/11$) substantially increase current levels supports the idea that current activation by OAG is more direct than activation by AVP.

Current inhibition by micromolar La³⁺

In the present study, the cation current endogenous to A7r5 cells was found to be completely blocked by 100 μM La³⁺ and Gd³⁺. Like current activation by OAG, the observed susceptibility to block by lanthanides at micromolar concentrations supports an involvement of the TRPC3/6/7 subfamily, rather than the TRPC4/5 subfamily in receptor-stimulated cation currents in A7r5 cells. TRPC4/5 currents have been shown to be augmented by La³⁺ at a concentration of 100 μM in three studies (Schaefer *et al.*, 2000; Strübing *et al.*, 2001; Schaefer *et al.*, 2002). By contrast, TRPC1 (Zitt *et al.*, 1996) and, more importantly TRPC3/6/7 channels (Zhu *et al.*, 1998; Kamouchi *et al.*, 1999; Okada *et al.*, 1999; Inoue *et al.*, 2001; Riccio *et al.*, 2002) have repeatedly been shown to be inhibited by application of lanthanides, with IC_{50} values ranging from 4 to 250 μM . It is not entirely clear why the values for half-maximal blocking concentrations obtained in independent studies for TRPC3 or TRPC6 vary by two orders of magnitude, as mentioned above. It should be noted, however, that reported IC_{50} values are consistently higher in Ca²⁺ imaging experiments than in electrophysiological recordings, suggesting the results might be influenced by the recording technique used. Shifts in the concentration dependence of inhibition by lanthanides may also result from the use of different species variants and from the cells used for heterologous expression.

Opposite effects of micromolar concentrations of both La^{3+} and Gd^{3+} on currents mediated by recombinant mTRPC5 and rTRPC6 were also found in the present study and were investigated in more detail. While whole-cell currents through TRPC6 were inhibited concentration-dependently by lanthanides, those through TRPC5 were stimulated by micromolar concentrations of the ion. These observations were extended to Gd^{3+} , which was approximately equally effective. The IC_{50} value obtained for inhibition of rat TRPC6 by La^{3+} was in good agreement with the values obtained in whole-cell patch clamp experiments for mouse TRPC6 (Inoue *et al.*, 2001) and human TRPC3 (Halaszovich *et al.*, 2000).

A novel finding of the present study was that higher lanthanide concentrations (≥ 1 mM) were less effective in stimulating, or even reversibly inhibited, currents carried by TRPC5. The dual effect of La^{3+} and Gd^{3+} on TRPC5 currents suggests that the lanthanides act at at least two distinct binding sites with different affinities.

Evidence for two different actions of lanthanides on the channel was supported by the effects of La^{3+} on single-channel currents in outside-out patches. La^{3+} caused a concentration-dependent decrease in single-channel current amplitude, while, at the same time, increasing the channel open probability (NP_o). Both effects were already observed at a concentration of $1 \mu\text{M}$. The concentration dependence of the increase in integral current ($i \times NP_o$) in outside-out patches closely paralleled the increase in whole-cell current, although the maximum stimulation was, on average, about 2- to 3-fold higher in outside-out patches than in whole-cell experiments. The reason for this is unclear, but may, at least partly, be related to the more effective buffering of Ca^{2+} in the excised patch than in whole-cell configuration. In support of this, stimulation by La^{3+} in whole-cell recordings was increased in a higher buffer capacity intracellular solution containing 30 mM BAPTA. Owing to the inability to resolve currents at millimolar concentrations of La^{3+} , it is not clear from the single-channel data why stimulation declines and inhibition occurs. Because of its weak concentration dependence, it seems unlikely that the decrease in single-channel current is solely responsible for the decrease in whole-cell current.

As mentioned above, stimulatory actions of 100 μM La^{3+} have been observed in whole-cell recordings for mouse, rat and human TRPC4- and TRPC5-mediated currents (Schaefer *et al.*, 2000; Strübing *et al.*, 2001; Schaefer *et al.*, 2002) and for receptor-operated currents in cells co-expressing mouse TRPC1 and mouse TRPC5 (Strübing *et al.*, 2001). In the latter, heteromultimers of TRPC1 and TRPC5 are thought to be formed, which, compared to homomeric TRPC5, have a drastically reduced single-channel current (-0.5 pA at a holding potential of -60 mV). Interestingly, in the above study, the single-channel amplitude was not affected by application of La^{3+} . For native nonselective cation currents, there is one report of a potentiation of the native current (I_{cat}) in rat ileal smooth muscle cells by La^{3+} , with an apparent K_d of 190 μM (Inoue *et al.*, 1998). From relaxation analysis, prolonged single-channel mean open life times were suggested to be the main cause of the augmentative effect of La^{3+} . Interestingly, in the mouse, TRPC4 is expressed in this tissue (Walker *et al.*, 2001). With regard to heterologously-expressed TRPC4 and TRPC5, it should be noted that some studies describe an inhibition by micromolar lanthanide concentrations (Okada *et al.*, 1998; McKay *et al.*, 2000). The reason for these differences is unclear.

Recently, Halaszovich *et al.* (2000) suggested that La^{3+} and Gd^{3+} block human TRPC3 channels from the cytosolic side of the membrane and that different apparent IC_{50} values might simply reflect different uptake rates for lanthanide ions in different cell types. From data obtained in the present study, there is little evidence in support of an intracellular site of action of the lanthanides on TRPC5 and TRPC6, though the possibility cannot be completely excluded. Both the inhibitory and stimulatory actions of the lanthanides had a fast onset and were very rapidly reversible. Furthermore, the effects on inward currents persisted and their time-course was unchanged when the buffer capacity, and possibly the speed of buffering increased, by using 30 mM BAPTA instead of 10 mM EGTA in the intracellular solution. However, it is noteworthy that outward currents through rTRPC6 were less susceptible to block by La^{3+} in BAPTA-containing pipette solution with respect to EGTA-containing solution.

Effects of $[Ca^{2+}]_o$ on current amplitudes

The current observed in A7r5 smooth muscle cells exhibits a dual dependence on $[Ca^{2+}]_o$. The current is partially inhibited by $[Ca^{2+}]_o$ in the physiological range, and the amplitude increases when $[Ca^{2+}]_o$ is decreased. On the other hand, complete removal of Ca_o^{2+} does not further potentiate the currents, but rather leads to a decrease in inward current amplitude. A similar complex dependence of cation currents on $[Ca^{2+}]_o$ has been reported for the noradrenaline-evoked current in rabbit portal vein smooth muscle cells (Helliwell & Large, 1996; Inoue *et al.*, 2001) and for store-operated currents in rat pulmonary artery (Ng & Gurney, 2001). For recombinant TRPC channels, an increase in current amplitude when $[Ca^{2+}]_o$ is raised above physiological levels has been described for recombinant mTRPC4/5 (Schaefer *et al.*, 2000). Okada *et al.* (1998) report that TRPC5 is stimulated by both intra- and extracellular Ca^{2+} . By contrast, hTRPC1 has been shown to be inhibited by external Ca^{2+} (Zitt *et al.*, 1996; Sinkins *et al.*, 1998; Lintschinger *et al.*, 2000). Variable effects of Ca_o^{2+} have been reported for TRPC3/6/7. Inhibitory effects of both extracellular and intracellular Ca^{2+} have been described for hTRPC3 (Kamouchi *et al.*, 1999; Lintschinger *et al.*, 2000; Zhang *et al.*, 2001). Likewise, increases in the amplitudes of whole-cell currents upon reduction of $[Ca^{2+}]_o$ from physiological levels to zero were described for mTRPC7 (Okada *et al.*, 1999). However, in contrast, another study on hTRPC3 (Zitt *et al.*, 1997) supports a stimulatory effect of extra- and intracellular Ca^{2+} on the currents mediated by this isoform. Moreover, expression of mTRPC6 in HEK293 cells was found to give rise to a receptor-stimulated current whose amplitude was markedly increased in Ca^{2+} -containing versus Ca^{2+} -free bath solutions (Inoue *et al.*, 2001).

To clarify the role of Ca_o^{2+} on channels of the TRPC4/5 and TRPC3/6/7 subfamily, the effects of changes in $[Ca^{2+}]_o$ on recombinant mTRPC5 and rTRPC6 currents were examined. The present study demonstrates differential regulation of TRPC6 and TRPC5 currents by Ca_o^{2+} . Whole-cell currents through TRPC6 were reversibly increased when $[Ca^{2+}]_o$ was decreased, whereas under the same conditions, currents mediated by TRPC5 were reversibly decreased.

In the present study, changing $[Ca^{2+}]_o$ from physiological levels to 200 μ M did not alter the single-channel amplitude, but increased the opening frequency of rTRPC6. By contrast, when $[Ca^{2+}]_o$ was increased to 20 mM, both single-channel amplitudes and opening frequency were significantly decreased. These findings contrast with a recent study on recombinant mTRPC6 (Inoue *et al.*, 2001), where a threefold increase in single-channel conductance was reported on increasing $[Ca^{2+}]_o$ from 0 to 1 mM. The reasons for these discrepant findings remain elusive.

It is unclear why the regulation of recombinant rTRPC6 by Ca_o^{2+} was not found to be bimodal although in the present study, TRPC6 was convincingly shown to be an essential component of the AVP-stimulated cation current in A7r5 cells. TRPC6 has also been shown to be important for the noradrenaline-stimulated cation currents endogenous to rabbit portal vein myocytes (Inoue *et al.*, 2001). As mentioned above, in these cells, endogenous currents were also found to be dually regulated by Ca_o^{2+} (Helliwell & Large, 1996). Large and colleagues (Helliwell & Large, 1996; Helliwell & Large, 1998; Aromolaran & Large, 1999; Albert & Large, 2001) investigated the mechanism of the dual effects of $[Ca^{2+}]_o$ on noradrenaline-evoked currents and found that the half-maximal $[Ca^{2+}]_o$ for the facilitory and inhibitory actions of $[Ca^{2+}]_o$ were markedly different, being 3 (Helliwell & Large, 1998) and 400 μ M (Helliwell & Large, 1996), respectively. Single-channel amplitudes of the cation currents endogenous to rabbit portal vein myocytes were found to be increased when $[Ca^{2+}]_o$ was increased from 0 to 3 μ M, but were constant for concentrations in the range of 3 μ M to 1.5 mM (Helliwell & Large, 1998; Albert & Large, 2001). It remains elusive why the regulation of recombinant TRPC6 by Ca_o^{2+} was not found to be bimodal in either the present study or the one by Inoue *et al.* (2001) and, more importantly, why the effects of $[Ca^{2+}]_o$ on rTRPC6 and mTRPC6 were found to be opposite in these studies. Perhaps regulation of recombinant TRPC6 is also dual, but some unknown regulatory factor can mask the dual dependence.

Single-channel current amplitudes

The similarity between recombinantly-expressed TRPC channel isoforms and the AVP-induced cation currents in A7r5 smooth muscle cells also includes the measured unitary

conductance of the current. The value of 30 pS at -60 mV determined by fluctuation analysis is comparable to those described for TRPC3, 4, 5 & 6 (Zitt *et al.*, 1997; Hurst *et al.*, 1998; Hofmann *et al.*, 1999; Kamouchi *et al.*, 1999; Schaefer *et al.*, 2000; Yamada *et al.*, 2000; Inoue *et al.*, 2001; Strübing *et al.*, 2001; Schaefer *et al.*, 2002; also see present study). It has to be noted, however, that noise analysis for the currents endogenous to A7r5 cells was performed in 200 μM $[\text{Ca}^{2+}]_o$ whereas the values determined in the above studies were obtained in physiological $[\text{Ca}^{2+}]_o$. Since the amplitudes of whole-cell currents in A7r5 cells are smaller in physiological $[\text{Ca}^{2+}]_o$ than in 200 μM Ca^{2+} -containing solution, the single-channel chord conductance may not be the same at physiological Ca^{2+} levels. However, in the present study, single-channel amplitude of recombinant TRPC6 was not found to be affected by reducing $[\text{Ca}^{2+}]_o$ from physiological levels to 200 μM . Moreover, Nakajima *et al.* (1996) reported comparable values (23 pS) for the mean slope conductance of the cation current endogenous to A7r5 cells in physiological $[\text{Ca}^{2+}]_o$.

An exclusive role for TRPC6 in receptor-activated cation currents in A7r5 cells

As shown above (compare also Table 3), similarities in the electrophysiological properties and the activation mechanism of recombinantly expressed TRPC3/6/7 and the AVP-induced currents in A7r5 smooth muscle cells strongly suggest that one or several members of the TRPC3/6/7 group are likely to form the endogenous cation channel expressed in A7r5 smooth muscle cells.

The cation currents endogenous to A7r5 cells were reversibly enhanced by flufenamate, which is an inhibitor of most nonselective cation channels (see e.g. Chen *et al.*, 1993; Popp *et al.*, 1993). Flufenamate has recently been shown to selectively increase currents mediated by recombinant mTRPC6, but to inhibit currents mediated by the other two members of the TRPC3/6/7 subfamily (Inoue *et al.*, 2001). The finding that the nonselective cation currents in A7r5 cells are enhanced by flufenamate, thus, provides evidence for the possible involvement of TRPC6 in agonist-induced cation currents in A7r5 cells.

Northern hybridization analysis demonstrated that *trpc6* is, indeed, transcribed in A7r5 smooth muscle cells. More importantly, of the structurally related isoforms TRPC3, -6

and -7 that respond to DAG, only mRNA coding for TRPC6 is expressed. In addition to TRPC6, mRNA for TRPC1 was detected, whereas no message for the isoforms TRPC2, -4 and -5 was found.

From the data on TRPC isoform expression, together with the activation of currents by OAG and the potentiating effect of FFA, it is likely that the AVP-activated, TRPC-like cation channels in A7r5 cells are formed by TRPC6. By contrast, the electrophysiological properties of the other expressed TRPC isoform TRPC1, i.e. its linear *I-V* relationship (Zitt *et al.*, 1996; Sinkins *et al.*, 1998; Lintschinger *et al.*, 2000) as well as its store depletion-dependent activation mechanism (Zhu *et al.*, 1996; Zitt *et al.*, 1996), are inconsistent with the properties of the nonselective cation currents in A7r5 cells. As mentioned in the introduction, functional channel complexes are believed to be composed of four TRPC proteins (cf. evidence provided for TRPV1, Kedei *et al.*, 2001), which implies possible formation of heteromeric channels when several TRPC proteins are co-expressed in the same cell. However, in A7r5 cells, a contribution of heteromers consisting of TRPC1 and TRPC6 to receptor-activated cation currents can largely be excluded because of a recent study by Hofmann *et al.* (2002), where TRPC3/6/7 are shown to exclusively assemble into homo- or heteromers within the confines of this subgroup of TRPC channels, but not to form heteromers with TRPC1, TRPC2 or TRPC4/5. A question that will have to be addressed in future studies concerns the role of TRPC1 in A7r5 smooth muscle cells.

The AVP-induced cation currents in A7r5 cells described in the present study show a striking similarity to receptor-activated cation currents in smooth muscle cells from the rabbit portal vein (Helliwell & Large, 1996; Helliwell & Large, 1997; Helliwell & Large, 1998). These similarities include e.g. *I-V* relation shape, single-channel conductance, dual dependence on $[Ca^{2+}]_o$ and PKC-independent activation by OAG. Recently, Inoue *et al.* (2001) provided convincing evidence that TRPC6 shows similar properties to, and is an important component of noradrenaline-stimulated cation channels in portal vein smooth muscle cells. In contrast to A7r5 cells, portal vein cells, however, also show significant levels of mRNA for TRPC3 and TRPC4, which may also form receptor-activated cation channels in these cells. Additional support for a

more general role of TRPC6 in vascular smooth muscle comes from a recent study by (Welsh *et al.*, 2002). The authors examined the nature of the channels that are responsible for the depolarization of rat cerebral arterioles observed in response to an increase in intraluminal pressure and proposed a major role for TRPC6 in the regulation of myogenic tone. Although the exact mechanisms by which pressure activates TRPC6 channels are not clear from the above study, pressure-induced activation of PLC (Osol *et al.*, 1993) is suggested to be essential. Moreover, TRPC6 was reported to be expressed in canine pulmonary and renal arteries (Walker *et al.*, 2001). Together with the results on A7r5 smooth muscle cells presented here, these results suggest that TRPC6 plays an important role in nonselective cation in different types of vascular smooth muscle cells. Preliminary findings on primary cultures of smooth muscle cells derived from neonatal and adult rat aorta showed the activation of a PLC-dependent cation current similar to the current observed in A7r5 cells. It has to be added, however, that in another smooth muscle cell type examined in the present study (a cell line derived from rat vena cava), no evidence for the presence of TRPC6 channels was found, suggesting that TRPC6 function may be restricted to certain vessel types.

A model for vasoconstrictor-dependent Ca^{2+} signalling in smooth muscle cells

A major question posed by the finding that TRPC6 is involved in the response of smooth muscle cells to vasoconstrictors is what the relative role of this channel is compared to other pathways that can contribute to an increase in $[Ca^{2+}]_i$. In Figure 39, a schematic view of the known components of vasoconstrictor-activated Ca^{2+} signalling in vascular smooth muscle cells is shown. In vascular smooth muscle cells, like in all muscle cells, Ca^{2+} is important for contraction (Jackson, 2000; Hill *et al.*, 2001) by regulating the interaction between actin and myosin filaments. Unlike the troponin-regulated mechanism of contraction and relaxation in striated muscle (Berchtold *et al.*, 2000), regulation of the myosin-actin interaction in smooth muscle cells involves Ca^{2+} -calmodulin-dependent stimulation of myosin light chain kinase and subsequent phosphorylation of the myosin light chain (Bolton *et al.*, 1999; Pfitzer, 2001; Sanders, 2001).

Vasoconstrictor-induced changes in $[Ca^{2+}]_i$ arise from release of stored Ca^{2+} , and Ca^{2+} influx across the plasma membrane. Besides ROCs like TRPC6, activated independently of store-depletion (Helliwell & Large, 1996; Broad *et al.*, 1999; Guibert & Beech, 1999; Koch & Korbmacher, 1999; this study; for review see McFadzean & Gibson, 2002), smooth muscle cells have also been shown to express store-operated channels (Noguera *et al.*, 1998; Broad *et al.*, 1999; McDaniel *et al.*, 2001; Ng & Gurney, 2001; Trepakova *et al.*, 2001) and voltage-gated Ca^{2+} channels. The latter are commonly dihydropyridine-sensitive L-type channels (Worley *et al.*, 1986; Benham *et al.*, 1987; Nelson & Worley, 1989; Ganitkevich & Isenberg, 1990; Matsuda *et al.*, 1990). Store-operated Ca^{2+} entry channels have repeatedly been implicated in the regulation of vascular tone (for reviews see Gibson *et al.*, 1998; Stevens, 2001; McFadzean & Gibson, 2002). However, the molecular nature of these channels and their exact role in vasoconstriction remain elusive. Interestingly, as is the case for receptor-activated Ca^{2+} -permeable channels gated independently of store-depletion, TRPC proteins have been suggested to be molecular components of native store-operated channels (Golovina *et al.*, 2001; Ng & Gurney, 2001; Xu & Beech, 2001). Hence, it is possible that different members of the same channel family contribute to both store-operated and store-depletion-independent Ca^{2+} entry.

Activation of voltage-gated Ca^{2+} channels in response to vasoconstrictors is dependent on the extent of vasoconstrictor-induced depolarization, which is determined by the interplay of ROCs and Ca^{2+} -activated Cl^- and K^+ channels. Interestingly, L-type VOCs, though shown to be present in A7r5 cells, were not found to contribute significantly to AVP-induced Ca^{2+} signalling in A7r5 smooth muscle cells. Previous studies on A7r5 and other aortic smooth muscle cells (Thibonnier *et al.*, 1991; Marchenko & Sage, 1993; Nakajima *et al.*, 1996; Broad *et al.*, 1999; Kawanabe *et al.*, 2001) also suggest that voltage-gated channels play only a minor role in vasoconstrictor-induced Ca^{2+} signalling in these cells. It is noteworthy, that some studies (Van Renterghem *et al.*, 1988; Guibert & Beech, 1999) even show clear inhibitory effects of vasoconstrictors on L-type VOCs. By contrast, studies on other types of vascular smooth muscle cells report membrane depolarization-mediated activation of VOCs in vasoconstrictor-dependent Ca^{2+} signalling (Goto *et al.*, 1989; Silberberg *et al.*, 1989; Nelson *et al.*, 1990; Chen & Rembold, 1995; Brock *et al.*, 1997; Davis & Hill, 1999).

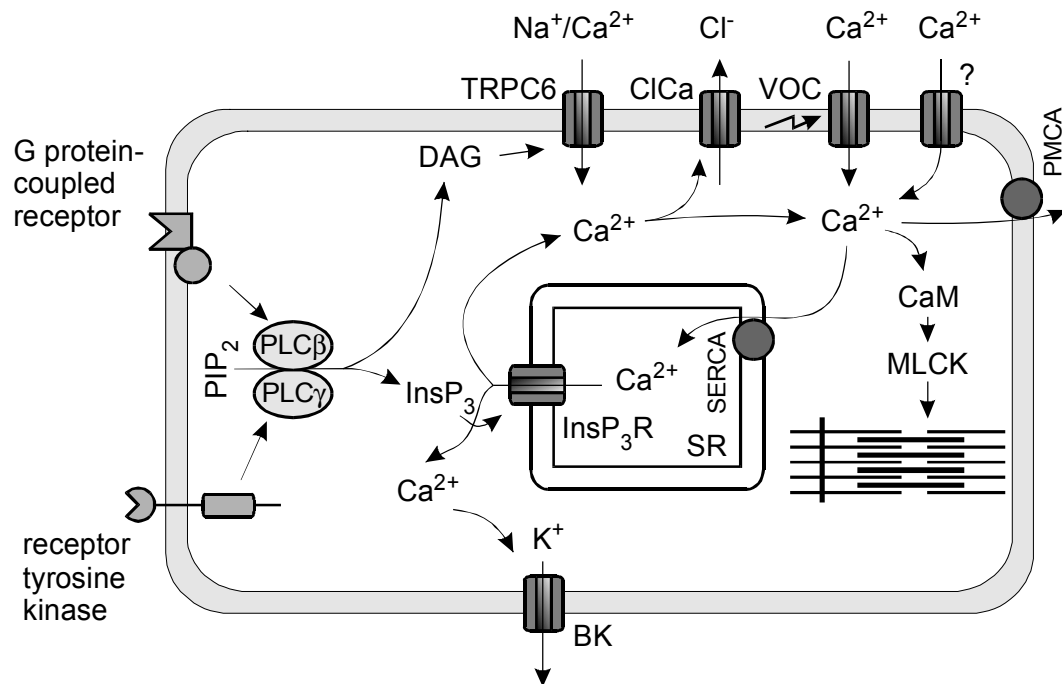


Fig. 39: **Phospholipase C-dependent Ca^{2+} -signalling in vascular smooth muscle cells.** Summary of the current knowledge of the components and pathways involved. BK: large-conductance Ca^{2+} -activated K^{+} channels, CaM: Ca^{2+} /Calmodulin, VOC: voltage-gated Ca^{2+} channels, ClCa: Ca^{2+} -activated Cl^{-} channels. MLCK: Myosine light chain kinase, SR: sarcoplasmic reticulum.

As mentioned above, an unresolved issue of vascular smooth muscle cell physiology is the relative contribution of these different Ca^{2+} entry pathways to vasoconstriction. Interestingly, Byron and colleagues (Byron, 1996; Fan & Byron, 2000; Li *et al.*, 2001; Byron & Lucchesi, 2002) report that low concentrations of vasopressin (10-500 pM) stimulate oscillations of $[\text{Ca}^{2+}]_i$ in A7r5 smooth muscle cells that are strictly dependent on extracellular Ca^{2+} and abolished by blockers of voltage-gated Ca^{2+} channels, whereas at higher AVP concentrations, Ca^{2+} influx through voltage-independent channel predominates (Broad *et al.*, 1999; this study). Accordingly, Moneer & Taylor (2002) show that the capacitative and non-capacitative Ca^{2+} entry pathways, described for A7r5 cells (Byron & Taylor, 1995; Broad *et al.*, 1999), are reciprocally activated and that only the latter operates during receptor activation (Moneer & Taylor, 2002). Hence, differential, context-specific co-ordination of the various Ca^{2+} entry pathways may be an important feature of smooth muscle cell function (Byron, 1996; Fan & Byron, 2000; Li *et al.*, 2001; Byron & Lucchesi, 2002; Moneer & Taylor, 2002).

Resumé and concluding remarks

From the combined electrophysiological and molecular biological data provided in the present study, TRPC6 is suggested to play a major role in receptor-stimulated cation currents in A7r5 smooth muscle cells.

Together with a recent report by Inoue *et al.* (2001), the present study for the first time demonstrates that an endogenous receptor-stimulated cation current shows properties identical to those described for TRPC isoforms in heterologous overexpression studies. In fact, both studies show that currents mediated by recombinant TRPC6 virtually mimic the behaviour of the native channels. In view of the concerns that have been raised with respect to the applicability of overexpression studies using single TRPC isoforms (Zitt *et al.*, 2002), the present study provides convincing evidence for the importance of these studies, in addition to other approaches, for elucidating the physiological role of TRPC channels. It also disproves the hypothesis proposed by Kiselyov & Muallem (1999) that activation of TRPC3/6/7 by DAG analogues is due to an overexpression artefact. According to their hypothesis, overexpressed channel proteins may become susceptible to atypical modulation when an equally large supply of the endogenous components of the 'true' signal transduction cascade is not available.

In future studies, the functional importance of TRPC6 channels for vasoconstriction will have to be evaluated. Ion channels formed by TRPC6 may be a potential target for novel drugs to alleviate hypertension or inhibit smooth muscle proliferation in arteriosclerosis.