Invited Review**Ca²⁺ entry channels involved in endothelin-1-induced contractions of vascular smooth muscle cells**

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Introduction

Endothelin-1 (ET-1) is a 21-amino-acid peptide and it is one of the most potent endogenous vasoconstricting agent yet discovered (Yanagisawa *et al.*, 1988). ET-1 binds to its receptors (typically ET_A receptor) on vascular smooth muscle cells (VSMCs) and subsequently induces an increase in the intracellular free Ca²⁺ concentration ([Ca²⁺]_i), which is essential for contraction of the cells. It is generally accepted that the major part of the ET-1-induced sustained contractions and increases in [Ca²⁺]_i requires the persistent entry of extracellular Ca²⁺ (Rubanyi and Polokoff, 1994; Komuro *et al.*, 1997; Zhang *et al.*, 1998). The earlier studies with patch-clamp technique have shown that voltage-operated Ca²⁺ channel (VOCC) is activated by ET-1 in VSMCs from porcine coronary artery (Goto *et al.*, 1989) and guinea-pig portal vein (Inoue *et al.*, 1990). However, subsequent studies showed that ET-1-induced contractions (Chabrier *et al.*, 1989; D'Orleans-Juste *et al.*, 1989; Turner *et al.*, 1989; Nakajima *et al.*, 1996; Komuro *et al.*, 1997) and increases in [Ca²⁺]_i (Huang *et al.*, 1990; Takuwa *et al.*, 1990; Zhang *et al.*, 1998) are virtually resistant to specific blockers of L-type VOCC such as nifedipine which completely suppress the contractions induced by depolarization with high K⁺ stimulation. Thus it is now considered that the ET-1-induced contractions and increases in [Ca²⁺]_i depend mainly on Ca²⁺ entry through channels other than VOCC. However, it is totally unknown what types of Ca²⁺ entry channel are involved in the contractions and increases in [Ca²⁺]_i induced by ET-1. This review focuses on the recent development of Ca²⁺ entry channels in VSMCs activated by ET-1.

Key words: receptor-operated Ca²⁺ channel, store-operated Ca²⁺ channel, endothelin-1, noradrenaline, TRP channel

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Receptor-operated Ca^{2+} channel (ROCC) and store-operated Ca^{2+} channel (SOCC)

ROCC

More than 20 years ago, it was suggested that receptor activation could lead to Ca^{2+} entry into smooth muscle cells by mechanisms independent of membrane depolarization, and the concept of receptor-operated Ca^{2+} channels (ROCCs) in smooth muscle was introduced (Bolton, 1979; van Breemen *et al.*, 1979). Therefore, at that time, ROCCs represented any plasma membrane Ca^{2+} permeable channels other than VOCC, which were opened as a result of the binding of an agonist to its receptor. Subsequent studies clearly showed that Ca^{2+} channels were opened following depletion of intracellular Ca^{2+} store with inhibitors of endoplasmic (ER) Ca^{2+} -pump ATPase, in the absence of receptor stimulation by agonists, and these Ca^{2+} channels were designated store-operated Ca^{2+} channels (SOCCs) (Putney, 1986; Putney, 1990). On the other hand, externally applied ATP were shown to activate Ca^{2+} -permeable nonselective cation channels in the rabbit ear artery (Benham and Tsien, 1987), which resulted from activation of P2X receptors (Khakh *et al.*, 2001). These channels form true ligand-operated ion channels in which the ligand (ATP) binding site and the ion channel are contained within the same macromolecule. These channels are now considered a separate entity from ROCCs. It follows that ROCCs are defined as: 1) channels molecules are separate from the ligand-binding protein; 2) they are activated by agonists acting on a range of G-protein-coupled receptors; 3) they are neither voltage-operated nor store-operated.

According to the definition, there seem to be a number of ROCCs. These can be differentiated mainly on the basis of mechanisms of channel activation (Fig. 1). It is important to note that the currents through ROCCs have all shown the properties of non-selective cation currents, with varying degrees of Ca^{2+} selectivity, and therefore the abbreviation ROCC may be more accurately defined as “receptor-operated cation channels”.

SOCC

Store-operated Ca^{2+} channels (SOCCs), also called capacitative Ca^{2+} entry channels, are defined as plasma membrane Ca^{2+} channels that are opened in response to a decrease in the concentration of Ca^{2+} in the lumen of the endoplasmic reticulum (ER) ($[\text{Ca}^{2+}]_{\text{er}}$). These are considered to be highly specific for Ca^{2+} . Under physiological conditions, the decrease in $[\text{Ca}^{2+}]_{\text{er}}$ is caused by the binding of IP_3 to its receptor in ER. The key event that initiates the opening of SOCCs is the decrease in $[\text{Ca}^{2+}]_{\text{er}}$ but not the Ca^{2+} released from ER. Therefore, store-depletion, regardless of how it is brought about, can activate SOCCs. Experimentally, this can be demonstrated using a variety of drugs all of which share the ability to deplete intracellular Ca^{2+} stores. Such drugs include the inhibitors of ER Ca^{2+} -ATPase pump such as thapsigargin and cyclopiazonic acid which, by inhibiting the active uptake of Ca^{2+} into ER, allow the stores to deplete “passively”, leading to receptor-independent activation of SOCCs.

Except in a very few cases, the membrane currents underlying store-operated Ca^{2+} entry have never been recorded, and hence have remained uncharacterized in detail. The current was first recorded in mast cells (Hoth and Penner, 1992, 1993) and was termed I_{CRAC} (Ca^{2+} -release activated current). This current was highly selective for Ca^{2+} with a single channel conductance

Fig. 1. The concept of receptor-operated Ca^{2+} channel (ROCC) and store-operated Ca^{2+} channel (SOCC). (A) ROCC: upper panel, intracellular-messenger-activated ROCC; lower panel, ROCC activated directly by G-protein. (B) SOCC.

of less than 1 pS. Difficulty in recording the current may be due to the fact that the currents are so small in comparison with their receptor-operated counterparts, with even the whole-cell store-operated current measuring little more than 10 pA.

Pharmacological properties of ROCCs activated by endothelin-1

Several researchers including us have shown that Ca^{2+} -permeable nonselective cation channel (NSCC) is activated by stimulation of native ET_A receptors in VSMCs (Van Renterghem *et al.*, 1988; Chen and Wagoner, 1991; Enoki *et al.*, 1995; Minowa *et al.*, 1997) and of recombinant human ET_A receptors expressed in *Ltk*⁻ cells, a mouse fibroblast cell line (Enoki *et al.*, 1995). "Nonselective" means that this channel is equally permeable to monovalent cations such as Na^+ and K^+ in the extra- and intracellular compartments. Importantly, this channel is considered to be permeable to Ca^{2+} , based on the finding that even when the bath solution of the whole-cell recording of the patch clamp contains only Ca^{2+} as a movable cation, an inward current is induced (Enoki *et al.*, 1995; Minowa *et al.*, 1997).

It has long been suggested that Ca^{2+} signaling mechanisms in response to ET-1 are different depending on its concentrations. Namely, higher concentrations of ET-1 stimulate both release of Ca^{2+} from intracellular stores via increased formation of IP_3 (Kasuya *et al.*, 1989; Rubanyi and Polokoff, 1994) and entry of extracellular Ca^{2+} , whereas lower concentrations of ET-1 stimulates only Ca^{2+} entry without Ca^{2+} release from intracellular stores (Enoki *et al.*, 1995). Thus it is possible that different Ca^{2+} entry channels are involved in the elevations of $[\text{Ca}^{2+}]_i$ induced by lower and higher concentrations of ET-1. To verify this possibility, the whole-cell currents induced by lower and higher concentrations of ET-1 were characterized in A7r5 cells (a cell line derived from rat thoracic aortic smooth muscle cells) (Enoki *et al.*, 1995; Minowa *et al.*, 1997; Iwamuro *et al.*, 1998; Iwamuro *et al.*, 1999).

Table 1. Pharmacology of three types of Ca^{2+} entry channels activated by lower (< 1 nM) or higher (> 10 nM) concentrations of ET-1

	Mn^{2+} permeability	Sensitivity	
		SK&F 96365	LOE 908
NSSC-1	–	–	+
NSSC-2	+	+	+
SOCC	?	+	–

NSSC-1 and NSSC-2: type 1 and 2 Ca^{2+} -permeable nonselective cation channel. SOCC: store-operated Ca^{2+} channel.

First it was confirmed that no significant currents through SOCC were induced following treatment with thapsigargin (Iwamuro *et al.*, 1999). In contrast, both lower and higher concentrations of ET-1 induced NSSC currents. Notably either concentration of ET-1 evoked currents in a bath solution containing only Ca^{2+} as a movable cation, indicating that NSSC is permeable to Ca^{2+} : $\text{PCa}^{2+}/\text{PCs}^{+}$ was calculated to be 2.1, based on the reversal potential of the current. The current at lower concentrations was abolished by replacement of Ca^{2+} in the bath solution with Mn^{2+} , indicating that the channel is impermeable to Mn^{2+} (Table 1). The effects of SK&F 96365 on the current was also tested. SK&F 96365 is well known as a blocker of the so-called receptor-mediated Ca^{2+} influx (Merrit *et al.*, 1990; Clementi and Meldolesi, 1996). Unexpectedly, the current at lower concentrations was resistant to SK&F 96365 (Table 1). Essentially similar results were obtained when the increase in $[\text{Ca}^{2+}]_i$ was used as an index.

In contrast, the current evoked by higher concentrations of ET-1 became partially sensitive to SK&F 96365, and abolished by combined treatment with Mn^{2+} and SK&F 96365 (Table 1) (Iwamuro *et al.*, 1998). These data show that two currents are activated by higher concentrations of ET-1: Mn^{2+} -sensitive and SK&F 96365-sensitive currents. The former component is considered to be the same as the current induced by lower concentrations of ET-1 and the latter is specific for higher concentrations of ET-1.

Another blocker of the so-called receptor-mediated Ca^{2+} influx, LOE 908 (Krautwurst *et al.*, 1993; Krautwurst *et al.*, 1994; Encabo *et al.*, 1996), was tested for inhibitory effects on currents through NSSCs induced by ET-1. Unlike SK&F 96365, LOE 908 was found to abolish the whole-cell currents induced by both lower and higher concentrations of ET-1 (Iwamuro *et al.*, 1999).

In summary, in addition to VOCC, ET-1 activates two types of Ca^{2+} -permeable nonselective cation channel in A7r5 cells depending on its concentrations (Table 1): lower concentrations of ET-1 activate one type of cation channel (designated NSSC-1), which is impermeable to Mn^{2+} , resistant to SK&F 96365 and sensitive LOE 908; higher concentrations of ET-1 activate another type of cation channel (designated NSSC-2) in addition to NSSC-1, which is permeable to Mn^{2+} , and sensitive to both SK&F 96365 and LOE 908. Importantly, it became possible to discriminate two types of NSSCs activated by ET-1 depending on their sensitivities to two drugs.

Pharmacological properties of SOCCs

Now that two types of NSSCs among Ca^{2+} channels activated by ET-1 can be discriminated

in terms of the sensitivities to SK&F 96365 and LOE908, pharmacological properties of SOCCs were analyzed. As describe above, because the currents though SOCCs could not be detected by whole-cell recordings, Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ in store-depleted cells was used as a measure of Ca^{2+} influx through SOCCs (Ca^{2+} depletion/ Ca^{2+} readdition protocol). That is, A7r5 cells were preincubated in the Ca^{2+} -free solution containing thapsigargin (an inhibitor of Ca^{2+} -pump in ER) (Thastrup *et al.*, 1990) or ionomycin (a Ca^{2+} ionophore) (Morgan and Jacob, 1994) to deplete the intracellular Ca^{2+} store, and subsequently Ca^{2+} was added to the solution. Under such conditions, the increase in $[\text{Ca}^{2+}]_i$ induced by addition of Ca^{2+} can be regarded as Ca^{2+} influx through SOCC. Indeed, the increase in $[\text{Ca}^{2+}]_i$ was resistant to nifedipine.

SK&F 96365 abolished the increase in $[\text{Ca}^{2+}]_i$ induced by addition of Ca^{2+} in A7r5 cells which had been preincubated with thapsigargin or ionomycin, indicating that SK&F 96365 is a blocker of SOCC (Table 1), as reported previously (Chung *et al.*, 1994; Franzius *et al.*, 1994). Taken together with the data on NSCCs as described above, these results show that SK&F 96365 is a blocker of NSCC-2 and SOCC without inhibitory effect on NSCC-1 (Table 1).

Notably, LOE 908 had no effect on the Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ in A7r5 cells depleted of the intracellular Ca^{2+} store as a measure of SOCCs (Iwamuro *et al.*, 1999), but it abolished both NSCC-1 and NSCC-2 activated by lower and higher concentrations of ET-1, as described above. Based on these data, it is concluded that LOE 908 is a blocker of NSCC-1 and NSCC-2 without inhibitory effect on SOCC (Table 1).

Several other members of blocker of the so-called receptor-mediated Ca^{2+} influx such as econazole, miconazole and clotrimazole (Clementi and Meldolesi, 1996) were also tested, and it was found that these drugs possess the same properties as SK&F 96365. In this context, LOE 908 is unique in that it has no effect on SOCC.

Using SK&F 96365 and LOE 908, it became possible to pharmacologically discriminate three voltage-independent Ca^{2+} channels (NSCC-1, NSCC-2 and SOCC) using LOE 908 and SK&F 96365 (Table 1). 1) NSCC-1 (activated by lower concentrations of ET-1) is sensitive to LOE 908 but resistant to SK&F 96365, 2) NSCC-2 (activated by higher concentrations of ET-1) is sensitive to both LOE 908 and SK&F 96365, and 3) SOCC is resistant to LOE 908 but sensitive to SK&F 96365.

Ca^{2+} entry channels involved in the ET-1-induced increases in $[\text{Ca}^{2+}]_i$ and contractions of rat aortic smooth muscle cells

Ca^{2+} entry channels involved in ET-1-induced contractions of rat thoracic aorta and increases in $[\text{Ca}^{2+}]_i$ in single VSMCs prepared from rat thoracic aorta were examined using SK&F 96365 or LOE 908 (Zhang *et al.*, 1999). The contractions and increases in $[\text{Ca}^{2+}]_i$ induced by either lower or higher concentrations of ET-1 depended on extracellular Ca^{2+} but were resistant to nifedipine. The responses to lower concentrations (< 0.1 nM) of ET-1 were abolished by either SK&F 96365 or LOE 908 alone (Fig. 2), indicating that the responses at lower concentrations of ET-1 involve only NSCC-2 (sensitive to both SK&F 96365 and LOE 908). The result that in rat aortic tissue, NSCC-2 is activated by low concentrations of ET-1 is in sharp contrast with that in A7r5 cells, where NSCC-1 is activated. The mechanism of discrepancy is at present unknown,

Fig. 2. Pharmacology of the contraction of rat thoracic aortic ring specimens and the increase in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of single vascular smooth muscle cells prepared from the aorta induced by a low concentration (10^{-10} M) of ET-1. At the indicated time, ET-1 was added to evoke contractions (left panels) and the increase in $[\text{Ca}^{2+}]_i$ (right panels). After the contraction and the increase have reached a steady-state, SK&F 96365 ($30 \mu\text{M}$) and LOE 908 ($30 \mu\text{M}$) were added. The contraction and the increase in $[\text{Ca}^{2+}]_i$ are sensitive to both SK&F 96365 and LOE 908, which is consistent with the property of NSCC-2.

but it might be the difference of phenotype (contractile form *vs.* proliferative form).

On the other hand, the responses to higher concentrations (> 1 nM) were suppressed by SK&F 96365 or LOE 908 to about 10% and 45% of control responses, respectively, and they were abolished by combined treatment with SK&F 96365 and LOE 908 (Fig. 3). The proportion of the LOE 908-resistant contraction (SOCCs) tended to increase with increases in the concentrations of ET-1. Based on pharmacological properties of Ca^{2+} -permeable channels, percent contribution of each channel was calculated. The calculation showed that the contractions and increases in $[\text{Ca}^{2+}]_i$ of rat aorta at higher concentrations of ET-1 involve NSCC-1, NSCC-2 and SOCC, which contribute 10%, 45% and 45%, respectively, to total Ca^{2+} entry (Fig. 4).

Comparison of Ca^{2+} entry channels in vascular smooth muscle cells activated by ET-1, noradrenaline and vasopressin

Now that pharmacological tools to analyze Ca^{2+} entry channels are available, we attempted to characterize and compare Ca^{2+} entry channels involved in contractions of rat thoracic aorta induced by ET-1, noradrenaline and arginine-vasopressin (AVP) (Fig. 4). Pharmacology of increases in $[\text{Ca}^{2+}]_i$ were also analyzed in single vascular smooth muscle cells (VSMCs) enzymatically prepared from the aorta (Furutani *et al.*, 2002).

A blocker of L-type VOCC (nifedipine) had no effect on the responses (contraction and increase in $[\text{Ca}^{2+}]_i$) induced by a high concentration of ET-1 (10 nM), but it suppressed the responses to NA and AVP to 75% and 65%, respectively, indicating that VOCC is not involved in the contraction by ET-1, whereas it contributes 25% and 35% to the contraction by NA and AVP,

Fig. 3. Pharmacology of the contraction of rat thoracic aortic ring specimens and the increase in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of single vascular smooth muscle cells prepared from the aorta induced by a high concentration (10^{-8} M) of ET-1. After the contraction and the increase have reached a steady-state, SK&F 96365 ($30 \mu\text{M}$) and LOE 908 ($30 \mu\text{M}$) were added. The LOE 908-resistant part (y) in the upper panel and SK&F 96365-resistant part (n) in the lower panel represents SOCC and NSCC-1, respectively. The proportion of NSCC-2 is calculated by subtracting n from x or y from z.

respectively. LOE 908 partially suppressed the nifedipine-resistant responses to ET-1 and AVP, but not those to NA, indicating that Ca^{2+} influx through NSCCs is involved in the contractions induced by ET-1 and AVP but not in those by NA. SK&F 96365 also partially suppressed the nifedipine-resistant responses to ET-1 and AVP, while it completely suppressed the responses to NA. LOE 908 in combination with SK&F 96365 abolished the nifedipine-resistant responses to either of the agonists. These results show that the contraction of rat aorta involves different Ca^{2+} influx entry channels depending on agonists (Fig. 4): (a) NSCC-1, NSCC-2, and SOCC for ET-1; (b) VOCC and SOCC for NA; and (c) VOCC, NSCC-1, NSCC-2, and SOCC for AVP.

Possible mechanisms for activation of NSCC and SOCC

To analyze the mechanism for activation of NSCCs and SOCCs by ET-1 in terms of G-proteins, we constructed a series of mutated ET_A receptors which specifically couple with one or two of the G proteins (Okamoto *et al.*, 1997; Kawanabe *et al.*, 2002a, 2002b). In one mutant, cysteine residues (385–388) in the C-tail of the receptor were replaced with serine (serine ET_AR): these residues are normally palmitoylated to be anchored to the plasma membrane (Okamoto *et al.*, 1997). In another mutant, the receptor was truncated just after the first cysteine residue ($\text{ET}_A\text{R} \Delta 385$). In the other mutant, the receptor was truncated just before these cysteine residues ($\text{ET}_A\text{R} \Delta 382$). Wild type ET_A receptors are coupled with Gq, Gs and G12 (Takagi *et al.*, 1995; Kawanabe *et al.*, 2002a, 2002b). Notably, serine ET_AR mutants retained the coupling with Gs and G12, but lost the ability to couple with Gq: $\text{ET}_A\text{R} \Delta 385$ retained the

Fig. 4. Percentage contribution of Ca²⁺ entry through voltage-operated Ca²⁺ channel (VOCC), type 1 and type 2 Ca²⁺-permeable nonselective cation channel (NSCC-1 and NSCC-2) and store-operated Ca²⁺ channel (SOCC) to the contraction of rat thoracic aortic ring specimens induced by ET-1 (10⁻⁸ M), noradrenaline (NA, 10⁻⁶ M) and arginine-vasopressin (AVP, 10⁻⁷ M). The percentages were calculated as described in the legend for Fig. 3.

coupling with Gq, but lost the ability to couple with Gs and G12: ET_AR Δ381 retained the binding of ET-1, but lost the coupling with Gs, G12 and Gq. These receptors were expressed in CHO cells and analyzed for the pharmacological properties of ET-1-induced increase in [Ca²⁺]_i of these cells, using SKF 96365 and LOE 908 (Kawanabe *et al.*, 2002b). In wild type ET_AR, ET-1-induced increase in [Ca²⁺]_i was partly suppressed by LOE 908 (NSCCs), and the remaining increase was abolished by SKF 96365 (LOE 908-resistant and SKF 96365-sensitive character corresponds to SOCCs), indicating that the increase is due to Ca²⁺ influx through NSCCs (NSCC-1 and NSCC-2) and SOCCs. In ET_AR Δ385, ET-1-induced increase in [Ca²⁺]_i was resistant to LOE 908, but abolished by SKF 96365 alone, indicating that the increase is due to Ca²⁺ influx through SOCCs. In serine ET_AR, transient increase in [Ca²⁺]_i was absent, and only sustained increase was observed. In this mutant, the increase was abolished by LOE 908, but resistant to SKF 96365, indicating that the increase is due to Ca²⁺ influx through NSCC-1. When the cells expressing wild type ET_ARs were pretreated with a PLC inhibitor (U73122), the same result was obtained as in serine ET_ARs. The effects of dominant-negative G12 on the increase in [Ca²⁺]_i were also tested: the ET-1-induced increase in [Ca²⁺]_i in cells expressing this G protein was sensitive to SKF 96365 but resistant to LOE 908, indicating that the increase is due to Ca²⁺ influx through SOCC.

Based on these data, it is concluded that 1) NSCC-1 is activated via a G12-dependent pathway; 2) NSCC-2 is activated via Gq/PLC- and G12-dependent pathway; 3) SOCC is activated via Gq/PLC-dependent pathway (Fig. 5).

Molecular biology of NSCC and SOCC

The most promising candidate molecules for SOCCs and ROCCs are a group of channel

Fig. 5. Hypothetical mechanisms for activation of type 1 and type 2 Ca^{2+} -permeable nonselective cation channel (NSCC-1 and NSCC-2) and store-operated Ca^{2+} channel (SOCC).

proteins designated *trp* (transient receptor potential) and *trpl* (*trp*-like), initially cloned from *Drosophila melanogaster* head cDNA library (Montell and Rubin, 1989; Phillips *et al.*, 1992). When expressed in insect Sf9 cells, *trp* forms a Ca^{2+} -selective channel activated by store depletion (Vaca *et al.*, 1994) and *trpl* forms a Ca^{2+} -permeable nonselective cation channel activated by IP_3 (Hu *et al.*, 1994; Dong *et al.*, 1995).

Mammalian TRP superfamily falls into one of the three major families on a phylogenetic basis (Montell *et al.*, 2002; Clapham *et al.*, 2003): the TRPC or canonical TRP family, with seven members (TRPC1-7), which are the most closely related to the original *Drosophila* TRP channels; the TRPV family, with six members (TRPV1-6) named after the first group member, vanilloid receptor; and the TRPM family with eight members (TRPM1-8), named after the original member, melastatin. Members of the TRP superfamily are believed to form NSCCs and SOCCs, but it is at present totally unknown which members are actually responsible for the channels. Because many papers have so far been published in the field of TRPC family, we focus on this family.

Based on structural and functional similarities, the TRPC family can be further subdivided into four different subfamilies: 1) TRPC1, 2) TRPC2, 3) TRPC3, 6 and 7, 4) TRPC4 and 5 (Vazquez *et al.*, 2004; Clapham *et al.*, 2003). By analogy to VOCC, it is likely that functional channels are formed by a tetrameric assembly of TRPC proteins which includes the likelihood of hetero-tetrameric assembly (Putney, 2004). Results, obtained by a number of groups using a variety of expression systems, have shown that all seven TRPC proteins, whether expressed alone or in combination, can form Ca^{2+} -permeable cation channels (Vazquez *et al.*, 2004; Spassova *et al.*, 2004). However, it has only rarely been possible to reproduce the phenotype of the Ca^{2+} entry pathway in a native cell by transfecting one or more of the TRPC genes into cultured cells. In particular, it has proved difficult to establish a consensus view regarding the activation mechanisms for these channels, because conflicting results are reported for almost every subfamily member on whether activation involves store-depletion or store-independent mechanisms. For example, TRPC3 was found to be store-operated in some studies (Vazquez *et*

al., 2001; Trebak *et al.*, 2002), but PLC-activated (probably by DAG) in others when expressed in HEK293 cells (Venkatachalam *et al.*, 2001). There is an exception to this chaotic situation. Several lines of evidence indicate that TRPC1 may be a component of SOCCs, or regulated by IP₃ receptors (Patterson *et al.*, 1999; Rosado *et al.*, 2000, 2001, 2002; Brownlow and Sage, 2003).

Regarding ROCC activation mode, it is now likely that a lipid mediator derived from PLC products (possibly the immediate breakdown product, diacylglycerol (DAG)) activates TRP. There is considerable evidence that activation of all TRPC channels is primarily mediated by activation of PLC. Recent studies indicate that DAG is the messenger for activation of at least some of the TRPC channels (TRPC 3, 6 and 7) (Hofmann *et al.*, 1999; Ma *et al.*, 2000; Okada *et al.*, 1999; Venkatachalam *et al.*, 2001; Zitt *et al.*, 2002), whereas other TRPC channels (TRPC 1, 4 and 5) do not respond to DAG (Hofmann *et al.*, 1999; Schaefer *et al.*, 2000; Venkatachalam *et al.*, 2003). Notably, this action of DAG is independent of PKC (Hofmann, *et al.*, 1999; Okada *et al.*, 1999). The mechanism of activation of TRPC 1, 2, 4 and 5 is not yet defined.

Although molecular entity of endogenous SOCCs is yet unidentified, activation mode of SOCCs have aroused the interest of many researchers in this field. According to the original capacitative Ca²⁺ entry model (Irvine, 1990; Berridge, 1995), SOCCs in the plasma membrane interact with IP₃ receptors in ER, resulting in physical coupling of Ca²⁺ release and Ca²⁺ entry. In fact, physical interactions between IP₃ receptors and every member of the TRPC family have been reported (Rosado *et al.*, 2000; Mery *et al.*, 2001; Tang *et al.*, 2001; Trebak *et al.*, 2003), but their physiological significance is uncertain. All members of TRPC family possess CIRB (CaM/IP₃ receptor binding) domain in the cytoplasmic C-tail, and this region may play a role in the interaction (Vazquez *et al.*, 2004).

In spite of a number of the puzzling reports as mentioned above, evidence is accumulating which suggest some members of TRPC family form ROCCs. For example, a thorough study by Inoue *et al.* (2001) strongly indicates that TRPC6 is an essential component of the NSCC activated by α -adrenoceptor. For this purpose, they compared the biophysical properties of an endogenous channel with those of TRPC6 expressed in HEK293 cells, and also analyzed the level of TRPC6 protein and the agonist-induced current after treatment with antisense oligonucleotides against TRPC6.

As described above, to clarify physiological roles of TRP channels is a difficult work. First, TRP channels are ubiquitously present among cell types, and multiple subtypes are expressed in most cells (Venkatachalam *et al.*, 2002; Clapham, 2003). Therefore, functional analysis of overexpressed channels is usually against a background of at least endogenous TRPs (and also ROCCs and SOCCs). Similarly, reduction of TRP channel expression by antisense oligonucleotides, siRNA or knock-out approaches is complicated by functional overlap and redundancy with multiple endogenous TRP channel subtypes. The more confusing puzzle is that for one member of TRP channels to be store-operated or non-store-operated might be dependent on expression levels of the channel protein: low levels of expression result in store-operated channel, whereas high levels of expression leads to disappearance of the original store-operated mode and appearance of receptor-operated mode (dependent on PLC activation, probably DAG) (Putney, 2004; Spassova *et al.*, 2004). Thus it seems to be a challenging work to clarify both molecular entities of ROCCs and SOCCs and their regulatory mechanisms.

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