Journal of Cellular Biochemistry 99:1503–1516 (2006)

Calcium Signaling in Non-Excitable Cells: Ca^{2+} Release and Influx Are Independent Events Linked to Two Plasma Membrane Ca^{2+} Entry Channels

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Abstract The regulatory mechanism of Ca^{2+} influx into the cytosol from the extracellular space in non-excitable cells is not clear. The ''capacitative calcium entry'' (CCE) hypothesis suggested that Ca^{2+} influx is triggered by the IP_3-mediated emptying of the intracellular Ca^{2+} stores. However, there is no clear evidence for CCE and its mechanism remains elusive. In the present work, we have provided the reported evidences to show that inhibition of IP_3-dependent Ca^{2+} release does not affect Ca^{2+} influx, and the experimental protocols used to demonstrate CCE can stimulate Ca^{2+} influx by means other than emptying of the Ca^{2+} stores. In addition, we have presented the reports showing that IP_3-mediated Ca^{2+} release is linked to a Ca^{2+} entry from the extracellular space, which does not increase cytosolic $[Ca^{2+}]$ prior to Ca^{2+} release. Based on these and other reports, we have provided a model of Ca^{2+} signaling in non-excitable cells, in which IP_3-mediated emptying of the intracellular Ca^{2+} store triggers entry of Ca^{2+} directly into the store, through a plasma membrane TRPC channel. Thus, emptying and direct refilling of the Ca^{2+} stores are repeated in the presence of IP_3, giving rise to the transient phase of oscillatory Ca^{2+} release. Direct Ca^{2+} entry into the store is regulated by its filling status in a negative and positive manner through a Ca^{2+}-binding protein and Stim1/Orai complex, respectively. The sustained phase of Ca^{2+} influx is triggered by diacylglycerol (DAG) through the activation of another TRPC channel, independent of Ca^{2+} release. The plasma membrane IP_3 receptor (IP_3R) plays an essential role in Ca^{2+} influx, by interacting with the DAG-activated TRPC, without the requirement of binding to IP_3. J. Cell. Biochem. 99: 1503–1516, 2006.

Key words: TRPC; Ca^{2+} influx; Ca^{2+} release; capacitative Ca^{2+} entry; IP_3; DAG; Stim1; Orai; thapsigargin

Calcium is a ubiquitous second messenger, regulating various cellular functions, such as proliferation, secretion, differentiation, and cellular movement. In resting cells, Ca^{2+} concentration in the cytosol ($[Ca^{2+}]_C$) is maintained at very low levels (55–100 nM). Stimulation of cells with an agonist, such as a hormones, cytokines, or growth factors, results in an increase in $[Ca^{2+}]_C$, which regulates the cellular functions. In non-excitable cells, Ca^{2+} signaling is initiated upon the activation of phospholipase C (PLC) signaling pathways [for review see Berridge, 1993]. Binding of an agonist to the cell surface receptor activates a PLC type (PLCγ or PLCβ), which in turn catalyzes the hydrolysis of phosphotidyl inositolbisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the release of Ca^{2+} from the IP_3-sensitive Ca^{2+} stores, causing a transient increase in $[Ca^{2+}]_C$. This is followed by a relatively sustained phase of increase in $[Ca^{2+}]_C$, due to the calcium influx from the outside. While the regulatory mechanism of Ca^{2+} release is relatively better understood, the mechanism of Ca^{2+} influx is in enigma. The "capacitative Ca^{2+} entry" (CCE) hypothesis proposed that Ca^{2+} influx into the cytosol is triggered by the emptying of the IP_3-sensitive calcium store [Putney, 1990]. However, till date the mechanism whereby the empty state of the Ca^{2+} store might induce Ca^{2+} influx remains elusive. More importantly, there is no direct experimental evidence to support this hypothesis. Alternatively, based on the detection of IP_3 receptor (IP_3R) in plasma membrane and the induction of inward Ca^{2+} current upon the intracellular administration of IP_3, it was proposed that Ca^{2+} influx into the cytosol is triggered by IP_3 [for review see Irvine, 1992].

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Received 23 July 2006; Accepted 25 July 2006

DOI 10.1002/jcb.21102

However, no IP_3-mediated Ca^{2+} conductance in plasma membrane, similar to Ca^{2+} conductance of IP_3R in Ca^{2+} stores, has been demonstrated [Mayrleitner et al., 1995].

On the other hand, a clear indication that the DAG or its metabolite(s), but not the IP_3-mediated Ca^{2+} release, triggers the Ca^{2+} influx came from the study of T-cell receptor (TCR)-driven Ca^{2+} signaling in T cells by Chakrabarti et al. [1995], although previous works have reported the existence of CCE in T cells [Premack et al., 1994]. Subsequently, Chakrabarti and Kumar [2000] provided direct evidence that naturally occurring endogenous DAG mediates T CR-driven Ca^{2+} influx, independent of protein kinase C (PKC) activation, and DAG lipase and DAG kinase pathways. This was followed by the similar findings in T cells and other cell types by other workers [for review see Bird et al., 2004].

Similar to T cells, in other cell types also a lack of dependency of Ca^{2+} influx on IP_3-mediated Ca^{2+} release has been reported, in many of which a CCE mechanism was reported before [Huang et al., 1991; Irvine, 1992; Lo and Thayer, 1993; Chakrabarti et al., 1995; Sasajima et al., 1997; Zitt et al., 1997; Liu et al., 1998; Trebak et al., 2003]. Thus, the findings centered on the mechanism of Ca^{2+} influx remain paradoxical. In the present study, we proposed a model of Ca^{2+} entry in non-excitable cells, to resolve this paradox. Our model accommodates all conflicting findings in a physiologically relevant way. This model is a detail version of a two-channel Ca^{2+} signaling model described by Chakrabarti et al. [1995]. In the present model, IP_3-mediated emptying of the intracellular Ca^{2+} store triggers entry of Ca^{2+} directly into the store, instead of into the cytosol, through a plasma membrane TRPC channel. Thus, in the presence of IP_3, the store is being emptied and directly refilled alternatively, giving rise to a transient phase of quantal oscillatory Ca^{2+} release. The other second messenger DAG activates another plasma membrane TRPC channel, leading to Ca^{2+} influx directly into the cytosol. IP_3R plays a role in DAG-induced Ca^{2+} influx, by interacting with the DAG-activated TRPC, without the requirement of binding to IP_3. Reported experimental evidences are provided here to support the present model. We also provide the reported findings to show that the experimental protocols used to support the CCE hypothesis can stimulate Ca^{2+} influx into the cytosol by means other than the emptying of the Ca^{2+} stores, thus indicating the improbability of CCE mechanism.

TRANSIENT RECEPTOR POTENTIAL CHANNELS

One of the milestones in our understanding of the Ca^{2+} signaling in non-excitable cells is the discovery of transient receptor potential (TRP) channels, which are permeable to various cations including Ca^{2+} and Mg^{2+}. The founding member of the TRP channels was discovered in *Drosophila*, while studying the mutants showing a TRP, instead of a sustained receptor potential, of photoreceptors in response to light [for review see Vazquez et al., 2004a; Pedersen et al., 2005]. Subsequently, two more TRP homologues, TRP-like (TRPL) and TRPγ, were discovered in *Drosophila*. We will restrict our discussion mostly to the mammalian homologues of TRP channel. Based on the sequence homology, the mammalian TRP channel super family can be divided into three main families—canonical TRP (TRPC), valinoid TRP (TRPV), and melastatin TRP (TRPM). There are four less characterized families, identified to be involved in several human diseases. There are seven members of TRPC (TRPC1–TRPC7), six members of TRPV (TRPV1–TRPV6), and eight members of TRPM (TRPM1–TRPM8). The Ca^{2+} influx channels are consisting of all TRPCs, TRPVs, and TRPM1, 2, 3, 6, 7, and 8 [Vazquez et al., 2004a; Pedersen et al., 2005].

Of all TRP channels, TRPC channels are most closely related to the *Drosophila* TRP. The TRPC members are grouped into four subfamilies—TRPC1, TRPC2, and TRPC3, 6, 7 and TRPC4–5 subfamilies. Members of TRPC family are activated upon the stimulation of the receptors that activate PLC signaling pathway, and hence are more relevant to the present context. Most of the mammalian cells express more than one type of TRPC, serving different physiological functions under the context of different cellular milieu. Structurally, all TRPCs contain six transmembrane domains (TM1–TM6) and include a pore region between TM5 and TM6. Members of TRPC are thought to assemble in homo and hetero-tetramer, largely within the confines of a subfamily [Strubings et al., 2003; Pedersen et al., 2005], perhaps to increase the functional diversity through the variations of cation selectivity and permeability. It has been shown that some TRPC channels (such as TRPC3, 6, 7) are permeable to Ba^{2+},

Mn^{2+}, and Sr^{2+}, while other members (such as TRPC1) are more selective for Ca^{2+} [Vazquez et al., 2004a; Pedersen et al., 2005]. However, in the physiological context all TRPC channels would serve as Ca^{2+} channels, because concentrations of Ba^{2+}, Mn^{2+}, and Sr^{2+} used in the experiments (2–5 mM) are much higher than their physiological concentrations. In the subsequent sections, we will discuss how various TRPC channels might be activated by PLC signaling events and pharmacological agents.

DIACYLGLYCEROL-MEDIATED Ca^{2+} INFLUX

Initial evidence showing that endogenous DAG or its metabolite may mediate native receptor-driven Ca^{2+} influx, was unfolded in the study of Chakrabarti et al. [1995]. They showed that PIP_2 hydrolysis, but not the IP_3-dependent Ca^{2+} release, is necessary for TCR-driven Ca^{2+} influx in T cells, indicating the involvement of DAG in this process. Later, Chakrabarti and Kumar [2000] provided direct evidence showing that naturally occurring endogenous DAG mediates TCR-driven Ca^{2+} influx in T cells, independent of PKC activation and metabolism of DAG through DAG lipase or DAG kinase pathway. These findings are in perfect agreement with the contemporary findings showing that the naturally occurring DAG and its analogues directly activate TRPC3, TRPC6, and TRPC7 expressed in mammalian cells [for review see Hofmann et al., 1999; Bird et al., 2004]. These initial findings were followed by a series of reports showing that exogenously added DAG analogues and naturally occurring DAG triggered Ca^{2+} influx in various cell types, independent of PKC activation and DAG metabolism by DAG lipase or DAG kinase [Hofmann et al., 1999; Okada et al., 1999; Ma et al., 2000; Zhang and Saffen, 2001; Gamberucci et al., 2002; Philipp et al., 2003; Trebak et al., 2003; Bird et al., 2004]. Till date, there is growing number of evidences showing that DAG stimulates calcium influx into the cytosol through the activation of several TRPC channels present in the plasma membrane. For example, several studies have shown that different cell types, stably transfected with TRPC proteins (TRPC3, TRPC6, and TRPC7), exhibited enhanced DAG-stimulated Ca^{2+} influx in a PKC independent manner [Vazquez et al., 2004a; Pedersen et al., 2005]. On the other hand, several studies have shown that TRPC1, TRPC4, and TRPC5 are not involved in DAG-stimulated Ca^{2+} influx [Hofmann et al., 1999; Pedersen et al., 2005]. Further, different mammalian cells have been shown to express one or more endogenous TRPC proteins (TRPC1–TRPC7). Some of these TRPC proteins (TRPC3, TRPC6, and TRPC7) are involved in DAG-mediated calcium influx [Vazquez et al., 2004a; Pedersen et al., 2005]. From these studies, it appears that TRPC3, TRPC6, and TRPC7 represent the principal DAG-activated Ca^{2+} channels in the plasma membrane. However, exception might be there. For example, some studies have shown that TRPC1 can be activated by DAG [Pedersen et al., 2005].

How DAG activates the TRPC channels is not clear. Mode of interaction between DAG and TRPC channels has not been demonstrated yet. However, it has been shown that DAG activates TRPC6 in a membrane delimited fashion, [Hofmann et al., 1999]. Further, it has been shown that tyrosine phosphorylation of TRPC3 and TRPC6, by src-tyrosine kinase (TK), is required for their activation by DAG [Vazquez et al., 2004b; Hisatsune et al., 2004].

Although DAG has been shown to activate TRPC3, TRPC6, and TRPC7, independent of PKC and DAG lipase, we cannot rule out yet that some metabolite(s) of DAG can activate some other TRP proteins. For example, the arachidonic acid (AA) has been shown to stimulate Ca^{2+} entry in many cell types [Bird et al., 2004]. The AA activates *Drosophila* TRPC channels and TRPC4 in mammalian cells [Bird et al., 2004]. Although, production of AA normally is the secondary result of the Ca^{2+}-dependent activation of cytoplasomic phospholipase A_2 [Bird et al., 2004], possibility remains that it may come from the degradation of DAG by DAG lipase. However, the major experimental evidences point mainly towards the activation of TRPC3, TRPC6, and TRPC7 by DAG.

CAPACITATIVE CALCIUM ENTRY

According to the "capacitative Ca^{2+} entry" (CCE) hypothesis, Ca^{2+} influx into the cytoplasm from the extracellular space is triggered by the IP_3-dependent emptying of the Ca^{2+} stores. Initially, it was suggested that Ca^{2+} enters into the cytosol through the empty Ca^{2+} stores, giving rise to the sustained increase in $[Ca^{2+}]_C$ [Putney, 1986]. Later, it was modified to suggest that the empty Ca^{2+} store triggers Ca^{2+} influx directly into the cytosol

[Putney, 1990]. However, it has been shown that blocking the IP_3-dependent Ca^{2+} release, using heparin, IP_3R blocker TMB8, anti-IP_3R antibody, and mutated IP_3R did not inhibit agonist-stimulated Ca^{2+} influx in the cells such as vascular smooth muscle cell [Huang et al., 1991; Lo and Thayer, 1993; Zitt et al., 1997; Liu et al., 1998; Davies-Cox et al., 2001; Trebak et al., 2003; Van Rossum et al., 2004]. Similarly, emptying of the ryanodine-sensitive Ca^{2+} store (overlapping with IP_3-sensitive store) did not induce Ca^{2+} influx in endothelial cells [Sasajima et al., 1997]. Paradoxically, in many of these cells CCE has been demonstrated by different experimental approaches [Premack et al., 1994; Sasajima et al., 1997].

The biggest difficulty with CCE hypothesis is the lack of direct experimental evidence to support it. This model was based on the stimulation of Ca^{2+} influx in the cells following the treatments that has potential to deplete the intracellular Ca^{2+} store. Several experimental approaches were taken to deplete the intracellular Ca^{2+} stores, such as using an inhibitor of microsomal Ca^{2+}-ATPase (thapsigargin being the most commonly used one), stimulating the cells with the agonist in Ca^{2+}-free medium, and incubating the cells in Ca^{2+}-free medium for a prolonged period of time [Irvine, 1992]. Another experimental strategy to support CCE was to use cell lines, in which IP_3R has been knocked down [Jayaraman et al., 1995]. However, there are experimental evidences to show that all of these strategies can affect Ca^{2+} entry into the cytosol independent of emptying of the Ca^{2+} stores, as will be discussed below.

Thapsigargin-Stimulated Ca^{2+} Influx

Since thapsigargin (TG) has been most widely used to inhibit microsomal Ca^{2+}-ATPase (Ca^{2+} pump) to demonstrate CCE, we will limit our discussion to this compound only. By inhibiting Ca^{2+} pump, TG prevents the reuptake of Ca^{2+} into the intracellular Ca^{2+} stores from the cytosol, leading to the depletion of the stores. Stimulation of non-excitable cells with TG in Ca^{2+}-free medium, followed by the removal of TG and readdition of Ca^{2+} to the medium, resulted in the increase in $[Ca^{2+}]_C$. This Ca^{2+} influx was assumed to be the Ca^{2+} influx stimulated by the empty Ca^{2+} stores, caused by TG treatment. However, no attempts were made to rule out the possibility that TG might induce Ca^{2+} entry into the cytosol by mechanism(s) different from its action on Ca^{2+}-ATPase. There are several reports which showed that TG-induced Ca^{2+} influx is distinctly different from agonist-induced Ca^{2+} influx. For example, it has been shown in many cells, such as glioma cells and $Itk^{-/-}$ T cells, that Ca^{2+} influx was triggered following the TG treatment, but not upon the emptying of IP_3-sensitive stores [Lo and Thayer, 1993; Liu et al., 1998]. In endothelial cells it has been shown that TG, but not emptying of the ryanodine-sensitive Ca^{2+} store by ryanodine/caffeine, evoked Ca^{2+} influx [Sasajima et al., 1997]. Furthermore, it has been shown that in DT40 cells PKC inhibited agonist-induced but not TG-induced Ca^{2+} influx [Venkatachalam et al., 2003], in rat prostate smooth muscle cells DAG stimulated Ca^{2+} influx even after TG-stimulated Ca^{2+} influx but not after agonist-evoked Ca^{2+} influx [Thebault et al., 2005], in platelets thrombin stimulated both Ca^{2+} and Ba^{2+} entry but TG evoked only Ca^{2+} entry [Hassock et al., 2002], and Gd^{3+} inhibited CCh-induced but not TG-induced Ca^{2+} influx in TRPC3-expressing (HEK)293 cells [Zhu et al., 1998]. Besides, Chakrabarti et al. [1995] found that the optimum concentration of TG required for maximum Ca^{2+} influx is 100 times higher than it is required for maximum Ca^{2+} release in T cells. If the empty store triggered Ca^{2+} release, then TG dose responsiveness should have been the same for both the Ca^{2+} release and influx. They also found that both the TG and anti-CD3 antibody evoked Ca^{2+} release to the same extent, but TG induced Ca^{2+} influx to a larger extent (30 times higher over basal $[Ca^{2+}]_C$) than anti-CD3 (2 times higher over basal $[Ca^{2+}]_C$). These results clearly showed that TG can induce Ca^{2+} influx by means independent of emptying the Ca^{2+} stores.

A multiplicity of cellular actions of TG has been discovered recently, one or more of which might account for the Ca^{2+} store-independent stimulation of Ca^{2+} influx by TG. It has been found that TG activates PLC, src- and syk-TK, and DAG production in many cells [Nofer et al., 1997; Nofer et al., 2000; Rosado et al., 2000; Dorval et al., 2003]. All of these can lead to increase in $[Ca^{2+}]_C$. For example, src family TK activates PLCγ1 signaling pathway linked to many receptors, such as TRC-CD3 complex in T cells [Fantl et al., 1993; Samelson, 2002]. Thus, TG-mediated Ca^{2+} influx in these cells could be due to the activation of PLCγ1. Activation of

phosphotidyl choline-specific PLC by TG results in the production of DAG [Nofer et al., 1997, 2000], which in turn would induce Ca^{2+} influx. This is further reinforced by the findings that TG-induced rise in $[Ca^{2+}]_C$ and the Ca^{2+} current are inhibited by the inhibition of PLC in platelets, lachrymal ascinar cell, basophilic leukemia cells, and B cells [Heemskerk et al., 1997; Broad et al., 2001]. Also, inhibition of TK has been found to inhibit TG-induced Ca^{2+} influx in fibroblast [Lee et al., 1993].

TG has been reported to modulate the function of many ion transport pathways in many cells. For example, TG has stimulating/enhancing effect on the reverse mode (Ca^{2+} influx/Na^+ efflux mode) of Na^+/Ca^{2+} exchanger in T cells and Chinese hamster ovary cells [Balasubramanyam et al., 1994; Chernaya et al., 1996]. It has also been found that TG activates inward cationic current carried out by Na^+ in pancreatic β cells, which in turn depolarizes the cells leading to Ca^{2+} influx through a voltage-sensitive Ca^{2+} channel [Curz-Curz et al., 2005]. It has also been shown that stimulation of T cells with TG or through TCR-CD3 complex activates voltage-dependent K^+ channel which provides an electrical driving force for Ca^{2+} influx [Feske et al., 2005]. Voltage-sensitive Ca^{2+} channels have been found to be essential for T-cell function [Chandy et al., 1984; Abdallah et al., 2005]. TG also induced Ca^{2+} influx in platelets by a Na^+-dependent mechanism [Kimura et al., 1993].

All the above findings clearly showed that inhibition of microsomal Ca^{2+}-ATPase is not the only action that TG exerts on cells. It exerts diverse actions on different cellular machinery, most of which has the potential to increase Ca^{2+} influx. Even we consider the inhibition of microsomal Ca^{2+}-ATPase is the only action of TG, yet we cannot rule out that TG-dependent rise in $[Ca^{2+}]_C$ is simply the result of entry of extracellular Ca^{2+} directly into the empty store followed by leakage from there due to the inhibited Ca^{2+}-ATPase. Thus, at present it is inappropriate to depict any TG-induced Ca^{2+} influx as store-operated Ca^{2+} influx.

Ca^{2+} Influx in Cells Pre-Stimulated in Ca^{2+}-Free Medium

Another approach to deplete the intracellular Ca^{2+} stores was to stimulate the cells with agonists in Ca^{2+}-free medium [for review see Irvine, 1992]. Subsequent removal of the agonists, followed by the readdition of Ca^{2+} in the medium, resulted in Ca^{2+} influx. It was concluded that during stimulation in Ca^{2+}-free medium, IP_3-sensetive Ca^{2+} stores were emptied, which triggered Ca^{2+} influx upon readdition of Ca^{2+}. It is not clear how these results lead to the conclusion, with certainty, that Ca^{2+} influx is triggered by emptying the Ca^{2+} stores. There could be other possibilities. One plausible explanation would be that Ca^{2+} influx pathway, once activated, remains open for a prolonged period of time in the absence of any Ca^{2+} influx. In this regard, it has been shown that stimulation of T cells through TCR/CD3 complex, under conditions in which IP_3-dependent Ca^{2+} release was partially or totally blocked, opened a Ca^{2+} influx pathway which remained active for an extended period of time in the absence of Ca^{2+} influx [Chakrabarti et al., 1995]. Similarly, prolonged opening of Ca^{2+} influx channel in avian nasal gland cells has been reported [Shuttleworth and Thompson, 1996]. The elevated levels of intracellular Ca^{2+} has a negative feedback effect on Ca^{2+} influx process and Ca^{2+} influx channels such as TRPC3, TRPC6, and TRPC7 [Muldoon et al., 1991; Tareilus et al., 1994; Lintschinger et al., 2000; Shi et al., 2004]. Thus, the persistent open state of the Ca^{2+} influx channel, in the absence of Ca^{2+} influx, is very likely due to the lack of a negative effect of increased level of intracellular Ca^{2+}.

Ca^{2+} Influx in the Cells Incubated for a Long Time in Ca^{2+}-Free Medium

It has been found that the addition of Ca^{2+} to the cells, incubated in Ca^{2+}-free medium for a long time, resulted in Ca^{2+} influx in the cytosol [Mason et al., 1991; Montero et al., 1991; Randriamampita and Tsien, 1993]. It was interpreted that the Ca^{2+} stores were depleted during the prolonged incubation in Ca^{2+}-free medium, which in turn triggered Ca^{2+} influx upon readdition of Ca^{2+} to the medium. However, it is not known whether such a treatment will always deplete the intracellular Ca^{2+} stores. It has been found that prolonged incubation of T cells in Ca^{2+}-free medium resulted in an increase in $[Ca^{2+}]_C$ upon readdition of Ca^{2+} in to the medium [Chakrabarti et al., 1995]. However, they found that such a treatment reduced only the basal $[Ca^{2+}]_C$, without reducing the Ca^{2+} contents of the Ca^{2+} stores. Thus,

it appears that Ca^{2+} influx in the cells, pre-incubated in Ca^{2+}-free medium, is triggered by lowered basal $[Ca^{2+}]_C$, not by the depleted Ca^{2+} stores. Later, it was found that in RBL-1 cells, lowering $[Ca^{2+}]_C$ up to 30 nM activated Ca^{2+} influx spontaneously, without any change in the Ca^{2+} contents of the stores [Krause et al., 1999]. These findings are reinforced by the reports that the Ca^{2+} channel TRPV6 is activated by lowered basal $[Ca^{2+}]_C$, when expressed in CHO-K1, HK293, and RBL cells [Yue et al., 2001; Bodding et al., 2002]. Similarly, another epithelial cell Ca^{2+} channel (ECC) has been found to be activated by lowering the basal $[Ca^{2+}]_C$ [Vennekens et al., 2000]. Thus, it appears that Ca^{2+} influx in the cells, pre-incubated in Ca^{2+}-free medium, is triggered by lowered $[Ca^{2+}]_C$, through the activation of a TRPV channel which are sensitive to intracellular osmolarity.

Ca^{2+} Influx in Cells Lacking IP_3R

Role of IP_3R in Ca^{2+} influx has been studied by knocking out or knocking down the IP_3R in many cells including T cells. It has been found that cells lacking IP_3R are deficient in agonist-induced Ca^{2+} release and influx [Jayaraman et al., 1995]. These findings apparently supported the CCE hypothesis. However, these findings were in contrary to the findings showing that blocking IP_3-dependent Ca^{2+} release did not inhibit agonist-induced Ca^{2+} influx [Huang et al., 1991; Lo and Thayer, 1993; Chakrabarti et al., 1995; Zitt et al., 1997; Liu et al., 1998; Davies-Cox et al., 2001; Trebak et al., 2003; Van Rossum et al., 2004]. This paradox was resolved by several other studies involving different mutant IP_3R and anti-IP_3R antibody. It was found that cells expressing N-terminus deleted IP_3R, which is defective in IP_3-binding and Ca^{2+} release function, lacks both the agonist-induced Ca^{2+} release and influx [Van Rossum et al., 2004]. However, the same cells expressing a C-terminus deleted IP_3R, which lacks Ca^{2+} channel function but not IP_3 binding, are defective in agonist-induced Ca^{2+} release without any impairment in Ca^{2+} influx [Van Rossum et al., 2004]. Furthermore, it has been shown that intracellular injection of anti-IP_3R antibody prevented IP_3 binding and Ca^{2+} release, without affecting Ca^{2+} influx [Davies-Cox et al., 2001]. From these findings it appears that an IP_3R with intact N-terminal domain, but not its IP_3-binding or Ca^{2+} conductance function, is essential for agonist-induced Ca^{2+} influx. Pertinent to this, it was further shown that IP_3R was required for DAG-evoked Ca^{2+} influx [Wedel et al., 2003; Hisatsune et al., 2005; Vazquez et al., 2006]. Thus, absence of Ca^{2+} influx in the cells lacking IP_3R does not indicate that Ca^{2+} influx is triggered by the IP_3-mediated emptying of the Ca^{2+} stores.

ENTRY OF Ca^{2+} FROM THE EXTRACELLULAR SPACE DIRECTLY INTO THE Ca^{2+} STORE

There are several reports showing that intracellular injection of IP_3 activates an inward Ca^{2+} current as revealed by patch clamp recordings [Philipp et al., 1996, 1998, 2000; Warnat et al., 1999; Kaznacheyeva et al., 2000]. Same Ca^{2+} current is also activated by TG in some cells [Philipp et al., 1996, 1998; Warnat et al., 1999]. A combination of molecular genetics and electro physiological approach revealed that the activation of Ca^{2+} current in the plasma membrane and increase in $[Ca^{2+}]_C$ followed by the application of TG or intracellular injection of IP_3, involved a group of TRPC channels, such as TRPC1, TRPC 4, and TRPC5 [Philipp et al., 1996; Warnat et al., 1999; Freichel et al., 2001; Mori et al., 2002]. However, as documented above, TG does not induce Ca^{2+} influx directly into the cytoplasm through the emptying of the Ca^{2+} stores. This rises the question as to the destination of Ca^{2+} entering through the TRPC channels activated by the emptying of Ca^{2+} stores by IP_3 or TG (from hereon we referred these TRPC as store-activated TRPC or SA-TRPC). To resolve the issue, we put forward that the Ca^{2+} from the outside ($[Ca^{2+}]_O$) enters into the empty Ca^{2+} stores directly through the SA-TRPC, thus refilling the stores. This process of emptying and refilling will continue in the continuous presence of a store emptying agent, causing an increase in $[Ca^{2+}]_C$. This explains why cells overexpressing TRPC1 showed enhanced TG-induced increase in $[Ca^{2+}]_C$ [Liu et al., 2000; Singh et al., 2000].

There are ample experimental evidences to support our postulation. First of all, it has been shown that IP_3-depenent Ca^{2+} release in T cells is controlled by the $[Ca^{2+}]_O$ through a plasma membrane Ca^{2+} entry pathway, which is pharmacologically different from the Ca^{2+} channel leading to Ca^{2+} influx into the cytosol

[Chakrabarti et al., 1995]. In addition to this finding, several workers have shown that there is a latency period before the first spike of Ca^{2+} release, due to the time required to buildup threshold levels of IP_3, after the application of agonist [Rooney et al., 1989; Kalthof et al., 1993; Berridge, 1994; Chiavaroli et al., 1994; Lee and Oliver, 1995; Wang et al., 1995]. Application of plasma membrane Ca^{2+} channel blocker, during this latency period, reduced the amplitude or totally inhibited the appearance of the first spike of Ca^{2+} release [Kalthof et al., 1993; Chiavaroli et al., 1994; Lee and Oliver, 1995; Wang et al., 1995]. Further, the latency period increased and the amplitude of the Ca^{2+} spike decreased or abolished at very low $[Ca^{2+}]_O$ [Rooney et al., 1989; Berridge, 1994; Lee and Oliver, 1995; McCarron et al., 2000]. Finally, no elevation of $[Ca^{2+}]_C$ was observed before this first spike or in between the spikes of Ca^{2+} release [McCarron et al., 2000]. Also, there are several reports showing that Ca^{2+} stores are refilled without the elevation of $[Ca^{2+}]_C$ [Byron et al., 1992; Madison et al., 1998; McCarron et al., 2000]. Besides, it has been shown that a Ca^{2+} pathway exists between extracellular space and lumen and refilling of the Ca^{2+} stores occurs by extracellular Ca^{2+} in the sub-plasmalemal space [Qian and Bourreau, 1999; Kang and Park, 2005]. These findings clearly showed that the entry of Ca^{2+} directly into the Ca^{2+} stores from the extracellular space exerts a regulatory effect on the IP_3-mediated Ca^{2+} release.

In order for the extracellular Ca^{2+} to enter the store directly, the stores must be in close vicinity to or physically associated with the plasma membrane and SA-TRPC. This has been clearly shown by several findings. First, cell fractionation study revealed the presence of IP_3R-containing ER membrane component tightly bound to the plasma membrane in the plasma membrane fraction [Rossier et al., 1991]. Second, electron microscopy, using the gold labeled anti-IP_3R monoclonal antibody, has detected and localized the IP_3R in the caveolar regions and small vesicular structures, just underneath the plasma membrane [Fujimoto et al., 1992]. These small vesicular structures were part of the ER. Third, cell fractionation study has detected one SA-TRPC (TRPC1) in the microsomal rather than in plasma membrane fraction [Hassock et al., 2002] indicating that TRPC1 is tightly bound to ER component. Further, IP_3R, TRPC1, and caveolin (a caveolar protein) has been found to be co-immunoprecipitated, indicating their close physical association [Lockwich et al., 2000; Singh et al., 2000; Trevino et al., 2001]. Similarly, TRPC4 has been found to be localized in caveolae [Torihashi et al., 2002]. These structural studies indicate a tight physical association between IP_3R-containing ER components, SA-TRPC, and plasma membrane at the caveolar regions of the plasma membrane.

Functional evidences also indicated that SA-TRPC are directly linked to the intracellular Ca^{2+} stores and regulates Ca^{2+} entry into the stores. It has been shown that knockdown of TRPC1 gene expression resulted in the reduced agonist-induced Ca^{2+} release and IP_3-induced Ca^{2+} current [Mori et al., 2002]. Not only that, knockdown of TRPC1 gene also reduced the affinity of IP_3R for IP_3 [Mori et al., 2002]. It has been found that the affinity of IP_3 for its receptor is decreased when the concentration of luminal Ca^{2+} ($[Ca^{2+}]_L$) is reduced [Oldershaw and Taylor, 1993] and the partially depleted Ca^{2+} stores are less sensitive to IP_3 [Missiaen et al., 1992]. Thus, a defect in the refilling of Ca^{2+} stores by extracellular Ca^{2+} might account for the reduced Ca^{2+} release and reduced affinity of IP_3R for IP_3 in TRPC1-defficient cells. These findings strongly indicated a functional link between extracellular Ca^{2+} and the Ca^{2+} stores through a SA-TRPC, such as TRPC1. Finally, another compelling evidence to show a functional link between the extracellular Ca^{2+} and IP_3-mediated Ca^{2+} release is that the depletion of Ca^{2+} stores by IP_3 induced a Ca^{2+} entry through caveolar regions, but not through any other region of the plasma membrane [Isshiki et al., 2002].

Based on the findings, discussed above, we postulate that agonist-induced emptying of the Ca^{2+} stores activates a SA-TRPC, through which the store is refilled directly by extracellular Ca^{2+}. This process of Ca^{2+} release and refilling will continue in the continued presence of a store-depleting agent (IP_3 or TG). Thus, a sustained rise in $[Ca^{2+}]_C$ would occur in the presence of TG, which would be reduced or diminished following the disruption of a SA-TRPC, such as TRPC1 [Mori et al., 2002]. The sustained rise of $[Ca^{2+}]_C$, induced by TG or intracellular dialysis with IP_3 [Liu et al., 2000; Singh et al., 2000], can easily be mistaken as the Ca^{2+} influx directly into the cytoplasm.

THE MODEL REGULATION OF Ca^{2+} SIGNALING BY TWO PLASMA MEMBRANE Ca^{2+} CHANNELS

The up-to-date reports presented above, clearly showed that emptying of Ca^{2+} stores activates Ca^{2+} entry from the extracellular space directly into the empty store, but not into the cytosol, through a plasma membrane TRPC channel. On the other hand, Ca^{2+} influx into the cytosol is activated by DAG through another plasma membrane channel. We now integrate all of these findings into a Ca^{2+} signaling model, which accommodates the findings which appeared contradictory before. The kernel of this model is the dependence of both IP_3-dependent Ca^{2+} release and DAG-activated Ca^{2+} influx on two different Ca^{2+} channels present in the plasma membrane. A simpler version of this model was described before by Chakrabarti et al. [1995].

Regulation of Ca^{2+} Release

In the present model (Fig. 1), we proposed that the IP_3-mediated emptying of the Ca^{2+} stores does not trigger Ca^{2+} influx into the cytosol. Rather, it triggers entry of Ca^{2+} from the extracellular space directly into the empty stores, through one of plasma membrane SA-TRPC linked to the Ca^{2+} stores. Once the store is refilled, IP_3 will act again to trigger the Ca^{2+}

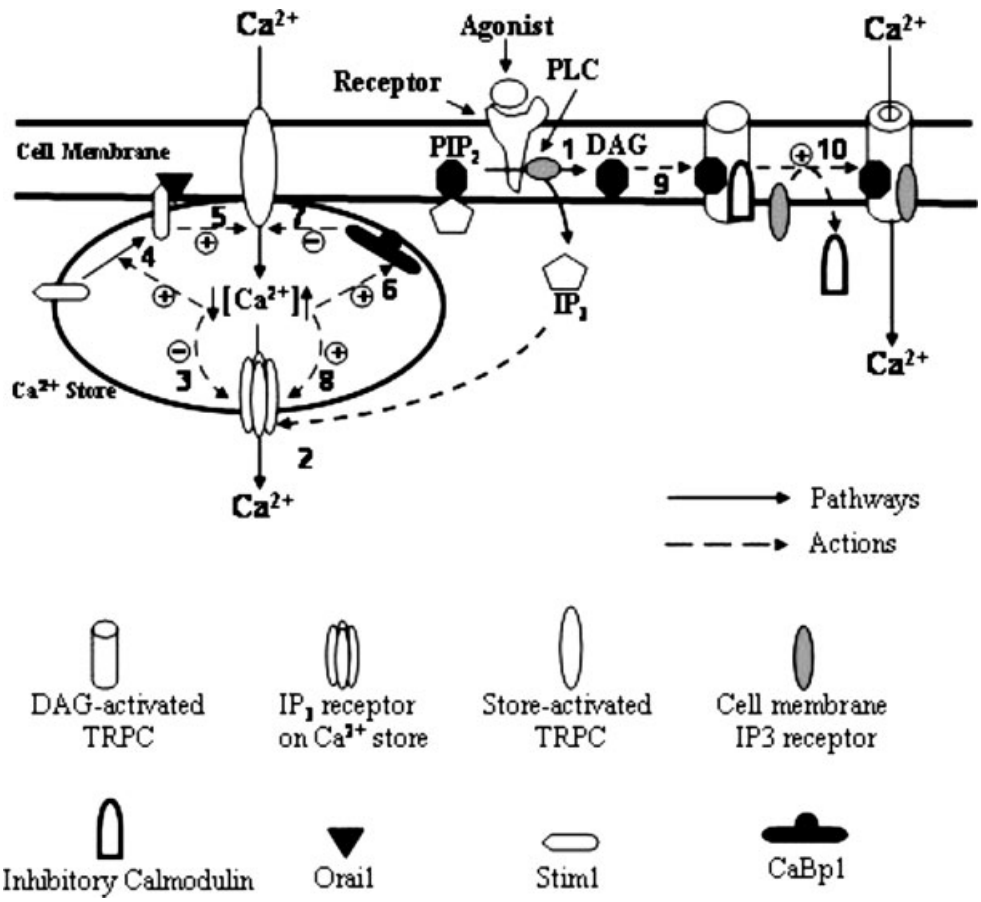

Fig. 1. Model of calcium signaling in non-excitable cells. Stimulation of the cells with agonists activates the PLC, which catalyzes the hydrolysis of PIP2 giving rise to IP_3 and DAG (1). IP_3 binds to its homo-tetrameric receptor on the intracellular Ca^{2+} stores and triggers Ca^{2+} release from the store (2). Reduced $[Ca^{2+}]_L$ renders IP_3R non-responsive to IP_3 (3). At the same time it triggers the translocation of Stim1 close to the inner surface of plasma membrane (4). There, Stim1 interacts with Orai and activates SA-TRPC (linked to the Ca^{2+} store), resulting in the direct refilling of the store by extracellular Ca^{2+} (5). Once the store is refilled, high $[Ca^{2+}]_L$ activates CaBp1 (6) which in turn shuts of the SA-TRPC (7). Simultaneously, high $[Ca^{2+}]_L$ makes IP_3R responsive to IP_3 (8), leading to another pulse of Ca^{2+} release if IP_3 is present. Repetition of this will lead to an oscillatory pattern of Ca^{2+} release. The other second messenger DAG binds to a DAG-dependent TRPC (9). This induces a conformational change in TRPC, which enables plasma membrane IP_3R to displace the inhibitory calmodulin from the TRPC, leading to the channel opening and Ca^{2+} influx into the cytosol from the extracellular space (10).

release. The process of Ca^{2+} release and refilling of the store will be repeated alternatively, in the presence of threshold level of IP_3. The functional significance of this process might be related to two important features of Ca^{2+} release. One feature is the oscillatory nature of Ca^{2+} release in which IP_3-mediated Ca^{2+} release occurs in repeated spikes, the frequency of which varies inversely with the agonist or IP_3 concentrations [Rooney et al., 1989; Kalthof et al., 1993; Berridge, 1994; Chiavaroli et al., 1994; Lee and Oliver, 1995; Wang et al., 1995]. The other feature of Ca^{2+} release is its quantal (all or none) nature, that is, there will be no Ca^{2+} release below or a maximal release above a threshold level of IP_3 [Rooney et al., 1989; Kalthof et al., 1993; Berridge, 1994; Chiavaroli et al., 1994; Lee and Oliver, 1995; Wang et al., 1995]. The regulatory mechanism of neither is clear. Our proposed alternation of Ca^{2+} release and direct refilling of the Ca^{2+} stores provides a simplest underlying mechanism of quantal oscillation of Ca^{2+} release. In order to achieve this, the concentration of the luminal Ca^{2+} ($[Ca^{2+}]_L$) must have the opposite regulatory effects on IP_3R and SA-TRPC. That is, IP_3R will be responsive to IP_3 and the SA-TRPC will be off when the Ca^{2+} store is completely filled with Ca^{2+}. The converse will be true when $[Ca^{2+}]_L$ decreases.

The regulatory effect of $[Ca^{2+}]_L$ on the IP_3R is supported by the fact that partially depleted Ca^{2+} stores are not sensitive to IP_3 and increase in $[Ca^{2+}]_L$ increased the affinity of IP_3R for IP_3 [Missiaen et al., 1992; Oldershaw and Taylor, 1993]. There are also findings indicating an inhibitory effect of $[Ca^{2+}]_L$ on SA-TRPC. Recently, a calcium-binding protein, CaBp1, has been found to bind TRPC5 and inhibit its activity [Kinoshita-Kawada et al., 2005]. Interestingly, CaBp1 has also been localized in ER lumen [Fullekrug et al., 1994]. Furthermore, it has been found that cells overexpressing CaBp1 has reduced IP_3-mediated Ca^{2+} release [Haynes et al., 2004]. These findings clearly indicate that $[Ca^{2+}]_L$ exerts an inhibitory effect on SA-TRPC, mediated through a CaBp, such as CaBp1.

Activation of SA-TRPC at reduced $[Ca^{2+}]_L$ might also involve a luminal Ca^{2+}-binding protein. Several findings in recent years have indicated the essential role of stromal interacting molecule 1 (Stim1), and Orai1 and Orai2 in the Ca^{2+} entry across the plasma membrane triggered by Ca^{2+} store depletion by TG or IP_3. Stmi1 is located in the ER with its Ca^{2+}-binding domain facing the ER lumen and Orai is located in the plasma membrane. Knocking down of Stim1 or Orai1 results in the reduction of store depletion-induced inward Ca^{2+} current and increase in $[Ca^{2+}]_C$ in many cells [Liou et al., 2005; Ross et al., 2005; Vig et al., 2006]. Interestingly, overexpression of Stmi1 or Orai alone caused very little or no enhancement in store-dependent Ca^{2+} entry; however, a dramatic enhancement was observed when both of them were overexpressed together, indicating a synergism between them [Zhang et al., 2005; Mercer et al., 2006; Peinelt et al., 2006; Soboloff et al., 2006]. Further, it has been found that when Ca^{2+} store is depleted, Stim1 is translocated to the punctate of ER in close apposition to the inner side of the plasma membrane [Liou et al., 2005; Zhang et al., 2005]. These findings clearly showed that a plausible mechanism exists through which lowered $[Ca^{2+}]_L$ can exert a stimulatory effect on SA-TRPC.

In one report, knocking down of Stim1 in T cells drastically decreased the anti-CD3-induced transient phase of Ca^{2+} release, without a significant effect on the sustained phase of $[Ca^{2+}]_C$ influx [Liou et al., 2005]. This indicated that Ca^{2+} release is dependent on a Stim1-regulated Ca^{2+} entry. Thus, based on all these recent reports we proposed a plausible mechanism of how $[Ca^{2+}]_L$ coordinates the oscillatory Ca^{2+} release and direct refilling of the stores by the extracellular Ca^{2+} (Fig. 1). In a nutshell, reduction in $[Ca^{2+}]_L$, as a result of Ca^{2+} release, will reduce the affinity of IP_3R for IP_3, inhibiting further Ca^{2+} release. At the same time, it will stimulate the translocation of Stim1 to the punctate where it will interact with Orai. The Stim1 and Orai will synergistically activate SA-TRPC, leading to Ca^{2+} entry into the store. Once the Ca^{2+} store is refilled, high $[Ca^{2+}]_L$ will activate the CaBp1 which in turn will shutoff the SA-TRPC. At the same time, high $[Ca^{2+}]_L$ will increase the affinity of IP_3R for IP_3 and render the store responsive to IP_3. If IP_3 is present, there will be another pulse of Ca^{2+} release. This cycle of Ca^{2+} release and store refilling will continue as long as threshold amount of IP_3 is present, giving rise to an oscillatory pattern of Ca^{2+} release.

Regulation of Ca^{2+} Influx

Immediately following the Ca^{2+} release, DAG triggers Ca^{2+} influx into the cytosol, through

the activation of a DAG-dependent TRPC channel, such as TRPC3, TRPC6, or TRPC7, resulting in a sustain increase in $[Ca^{2+}]_C$. The DAG activates these TRPC channels independent of PKC activation and metabolism of DAG by DAG lipase or DAG kinase. However, there are several reports showing that agonist and DAG-induced Ca^{2+} influx requires the interaction of the TRPC channels with IP_3R [Kiselyov et al., 1998, 1999; Boulay et al., 1999; Davies-Cox et al., 2001; Lockwich et al., 2001; Zhang et al., 2001; Van Rossum et al., 2004; Vazquez et al., 2006]. This might be the reason why onset of Ca^{2+} influx slightly lags behind the onset of Ca^{2+} release, although IP_3 and DAG are produced simultaneously.

The nature of interaction between IP_3R, DAG, and DAG-dependent TRPC is not clear. A physical association between the N-terminal domain of IP_3R (IP_3-binding domain) and TRPC3, TRPC6, and TRPC7 has been demonstrated [Boulay et al., 1999]. Regulatory effect of Ca^{2+}-calmodulin on TRPC6 activity, and the interaction of TRPC6 and TRPC3 with calmodulin and IP_3R have been demonstrated [Boulay, 2002; Zhu and Tang, 2004]. Further, it has been shown that an inhibitory calmodulin is bound to TRPC3 at a site which overlaps with its IP_3R-binding site, and TRPC is activated through the displacement of this inhibitory calmodulin by the IP_3R [Boulay et al., 1999]. This displacement must occur only in the presence of DAG, because these TRPCs are activated only in the presence of DAG. Thus, we proposed in the present model that binding of DAG to TRPC causes conformational changes in the channel, which enables the IP_3R to remove the inhibitory calmodulin, leading to the channel opening (Fig. 1). However, interaction between TRPC channels and the IP_3-binding domain of IP_3R will interfere with IP_3 binding to its receptor and Ca^{2+} release. This problem can be overcome by an IP_3R which will have easy access to TRPC, and that could be accomplished by the IP_3R present in the plasma membrane [Khan et al., 1992]. The function of the plasma membrane IP_3R was enigmatic, because of its poor Ca^{2+} conductance activity perhaps due to its binding to PIP2 of plasma membrane [Mayrleitner et al., 1995; Lupu et al., 1998]. Thus, we proposed in our model (Fig. 1) that it is the plasma membrane IP_3R which interact with and regulates DAG-dependent TRPC, sparing the IP_3R of the Ca^{2+} stores for its IP_3-dependent Ca^{2+} release function.

DISCUSSION

The model of Ca^{2+} signaling in non-excitable cells we presented above has three crucial features. One feature is the dependence of the IP_3-mediated Ca^{2+} release on $[Ca^{2+}]_O$, such that the empty store is refilled by Ca^{2+} directly from the extracellular space through SA-TRPC. The refilled store will prompt another pulse of Ca^{2+} release if IP_3 is present. We have postulated that the repetition of Ca^{2+} release followed by refilling of the store in the presence of IP_3 serves as the simplest underlying mechanism for two fundamental properties of Ca^{2+} release, all-or-none (quantal) and oscillatory Ca^{2+} release. To enable this mechanism operational, a SA-TRPC in the plasma membrane must be in contact with IP_3-dependent Ca^{2+} stores. Besides, a completely filled Ca^{2+} stores must have a positive effect on IP_3R and a negative effect on SA-TRPC. Conversely, an empty Ca^{2+} store will have a negative effect on IP_3R and a positive effect on SA-TRPC. We have provided reported findings to support this and a mechanism whereby this can be achieved. To make the role of CaBp1 clearer, its specific localization in Ca^{2+} stores in various cell types, inhibition of other SA-TRPCs by CaBp1, inhibition of IP_3-mediated Ca^{2+} release by CaBp1 in various other cell types, and nature of interaction between SA-TRPC and CaBp1 need to be determined. It would be also interesting to know if there are other proteins similar to CaBp1. Similarly, studying the mechanism of translocation of Stim1, nature of interaction between Stim1 and Orai, and the involvement of SA-TRPC in Stim1/Orai-dependent Ca^{2+} entry will greatly facilitate our understanding in the role of Stim1 and Orai in signaling the empty state of the Ca^{2+} store to the activation of SA-TRPC. This can be achieved by studying the effect of knocking down the Stim1 and Orai on Ca^{2+} entry in the context of SA-TRPC, and nature of interaction between SA-TRPC and Stim1/Orai.

Another critical aspect of our model is that DAG binds to DAG-dependent TRPC, which causes conformational changes in TRPC enabling IP_3R to displace inhibitory calmodulin from TRPC, leading to channel opening. The first step to confirm this would be to

demonstrate the binding of DAG to TRPC. The next step would be to demonstrate if IP_3R interaction with TRPC is dependent on or modulated by DAG binding to TRPC. Although interaction between IP_3R and TRPC has been demonstrated in un-stimulated cell lines over-expressing TRPC, it would be crucial to know if interaction of IP_3R with the endogenous TRPC is triggered or facilitated by agonist stimulation, because quite often heterologously expressed TRPC shows significant activity in the absence of any stimulation [Zhu et al., 1998], the reason of which is not clear. The third crucial future of the present model is that plasma membrane IP_3R, but not the Ca^{2+} store IP_3R, acts as the scaffold to interact with DAG-dependent TRPC. The simple way to determine this would be to examine if IP_3R and a DAG-activated TRPC are co-immunoprecipitated from the highly purified plasma membrane fraction (without any microsomal contamination), taken from the cells treated with agonist, DAG or untreated.

Another important question in Ca^{2+} signaling centers around the functional significance of two sources of increase in $[Ca^{2+}]_C$, that is, release from the store followed by the influx from outside. Although the role of Ca^{2+} influx in different cellular functions is well known, enigma remains regarding the role of Ca^{2+} release. We have shown that the process of Ca^{2+} release, not the released Ca^{2+} itself, is essential for T-cell proliferation [Kumar and Chakrabarti, 2000], indicating that transient changes in $[Ca^{2+}]_L$ or changes in IP_3R during Ca^{2+} conduction, triggers some processes essential for cell activation. Finally, like Ca^{2+} release, Ca^{2+} influx also oscillates, the regulatory mechanism of which is yet to be determined. Also the functional significance of oscillation of Ca^{2+} signaling is to be determined.

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