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Phospholipase C Signaling and Calcium Influx

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Introduction

Calcium signaling is a process whereby the extremely low cytoplasmic Ca^{2+} concentration increases in a deliberate and specific manner to trigger downstream cellular events. Virtually every tissue, and even every cell type in the human body utilizes some form of Ca^{2+} signaling to function or survive. There are many mechanisms which can generate Ca^{2+} signals; perhaps the most widely encountered, and the focus of this review, is a mechanism involving a lipid metabolizing enzyme, phospholipase C.

Phospholipase C is a truly remarkable signalling moiety. We know of no other single enzyme that can produce (or modulate), directly, three distinct signals: inositol 1,4,5-trisphosphate (IP_3), diacylglycerol, and phosphatidylinositol 4,5-bisphosphate (PIP2). The association of phospholipase C with Ca^{2+} signaling dates back arguably to experiments published by Lowell Hokin (Hokin, 1966) establishing the relative independence of inositol lipid turnover from Ca^{2+} signaling. It was just this independence from Ca^{2+} signaling that suggested to Bob Michell that inositol lipid turnover, initiated by phospholipase C-mediated inositide breakdown, was upstream of Ca^{2+} signaling (Michell, 1975; Michell *et al.*, 1977). It was later established that the initiating reaction was the breakdown of the relatively minor inositol lipid, PIP2 (Creba *et al.*, 1983; Weiss *et al.*, 1982; Berridge, 1983), leading Mike Berridge (Berridge, 1983) to propose that the released head group, IP_3 , could function as a Ca^{2+} signaling second messenger. This was rapidly confirmed experimentally by Berridge and his collaborators (Streb *et al.*, 1983), and subsequently by other investigators (Burgess *et al.*, 1984; Prentki *et al.*, 1984; Joseph *et al.*, 1984; Hirata *et al.*, 1984; Biden *et al.*, 1984; Whittaker & Irvine, 1984; Suematsu *et al.*, 1984; Brown & Rubin, 1984).

All three of the three phospholipase C derived signals regulate ion channels. Numerous ion channels require PIP2 for optimal activity and thus can be negatively regulated by phospholipase C activity (Suh & Hille, 2005; Hilgemann *et al.*, 2001). The IP_3 receptor in an intracellular calcium permeable channel activated by the phospholipase C product, IP_3 (Mikoshiba, 2007). However, this review will focus primarily on plasma membrane channels that are activated as a consequence of phospholipase C activation. There are two major categories of such channels, the store-operated channels, and the TRPC channels.¹

Phospholipase C and Store-operated Channels

In order to understand how phospholipase C and IP_3 function to regulate Ca^{2+} signaling, it is necessary to understand the major and distinct modes by which Ca^{2+} signals are generated.

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Basically, the two general processes are release from intracellular stores, and influx of Ca^{2+} across the plasma membrane (Bohr, 1963; Putney *et al.*, 1981). These two processes have been known to be linked in some way long before the nature of that linkage was known (Putney, 1977). The cytoplasm of eukaryotic cells contains, under resting conditions, about 100 nM $[\text{Ca}^{2+}]_i$. The extracellular Ca^{2+} concentration is of the order of 1–2 mM, and the concentration in the major intracellular storage compartment, the endoplasmic reticulum, is estimated to be only a bit lower, in the range of 0.1–1.0 mM (Bygrave & Benedetti, 1996). The Ca^{2+} signaling function of IP_3 was attributed to the release of Ca^{2+} stores in the endoplasmic reticulum; indeed, the nature of the initial experiments, utilizing either microsomal fractions or cells whose plasma membranes had been permeabilized to allow access of the inositol phosphate, precluded investigation of mechanisms of Ca^{2+} influx. However, subsequent experiments did in fact reveal that IP_3 could also produce an activated influx of Ca^{2+} across the plasma membrane (Slack *et al.*, 1986; Bird *et al.*, 1991). Yet, the preponderance of evidence suggested that this influx was not due to a direct action of IP_3 at the plasma membrane. First, while IP_3 readily released Ca^{2+} from microsomal fractions derived from endoplasmic reticulum, it did not do so from plasma membrane vesicles (Ueda *et al.*, 1986). Reloading of intracellular stores with Ca^{2+} , after cessation of the phospholipase C-linked stimulus, occurred just as rapidly as when the stimulus was present (Parod & Putney, 1978; Casteels & Droogmans, 1981). These observations led to a proposal whereby the activation of Ca^{2+} influx occurred secondarily to the depletion of the endoplasmic reticulum Ca^{2+} pool (Putney, 1986). The more definitive proofs of this concept came but a few years after. First, by use of the newly developed chemical Ca^{2+} indicators developed by Roger Tsien (Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1986) it was shown that Ca^{2+} channels did in fact remain open well after removal of the phospholipase C-linked stimulus, as long as the intracellular Ca^{2+} stores remained empty (Takemura & Putney, 1989). Second, the discovery of the highly specific inhibitor of the sarcoplasmic- endoplasmic reticulum Ca^{2+} ATPase, thapsigargin, afforded an experimental paradigm whereby Ca^{2+} stores could be depleted without activation of surface receptors or phospholipase C (Jackson *et al.*, 1988; Thastrup *et al.*, 1990). Application of thapsigargin to isolated parotid acinar cells activated Ca^{2+} influx, and this influx was not additive with influx activated through the phospholipase C-coupled muscarinic receptors (Takemura *et al.*, 1989). This process by which the loading status of the endoplasmic reticulum regulates plasma membrane Ca^{2+} influx was initially called *capacitative calcium entry* because of the analogy with electrical circuitry wherein a resistance (plasma membrane) and capacitance (Ca^{2+} store) in series mutually regulate one another. However, today perhaps the more popular term is *store-operated calcium entry* occurring through *store-operated calcium channels*.

Research over the ensuing twenty years or so focused on attempts to understand the signal between endoplasmic reticulum and plasma membrane, as well as the nature of the channels themselves. A notable advance came in 1992 with the first report by Hoth and Penner (Hoth & Penner, 1992) of a membrane current activated by Ca^{2+} store depletion. Interestingly, this current has been described in an earlier report from another laboratory (Lewis & Cahalan, 1989) but it was not recognized as such. Hoth and Penner (Hoth & Penner, 1992) called the current *calcium release-activated calcium current* or I_{CRAC} . The more precisely defined biophysical properties of I_{CRAC} provided a rigorous signature for future work examining the molecular basis for this signaling pathway. I_{CRAC} was shown to be a highly Ca^{2+} selective current, such that the current-voltage relationship is highly inwardly rectifying with a positive reversal potential (Hoth & Penner, 1992; Hoth & Penner, 1993). As is the case for other Ca^{2+} -selective channels, Ca^{2+} both blocks as well as permeates the channels such that severe reduction of external divalent cations produces larger inward Na^+ currents (Hoth & Penner, 1993). These monovalent cation currents are transient, however, because of a secondary augmenting effect of external Ca^{2+} (Zweifach & Lewis, 1996). Perhaps one of the most interesting properties of the CRAC channels is their apparent extremely small unitary

conductance. Hoth and Penner were not able to detect single channel events by conventional patch-clamp techniques, and their size was estimated later by use of noise analysis to be of the order of a few tens of fS (Zweifach & Lewis, 1993).

The *Drosophila* photoreceptor responds to light by activation of a Ca^{2+} permeable channel regulated by phospholipase C, termed TRP (Hardie & Minke, 1993). TRP stands for transient receptor potential, a description of the electrophysiological phenotype when the gene encoding TRP is mutated (Cosens & Manning, 1969). Because TRP is a Ca^{2+} channel activated downstream of phospholipase C, conjecture arose that it might represent the long-sought store-operated Ca^{2+} channel (Hardie & Minke, 1993). When a family of mammalian homologs was discovered, speculation continued and initial experimental results were indeed consistent with such a conclusion (Zhu *et al.*, 1995; Zhu *et al.*, 1996; Kiselyov *et al.*, 1998). However, this idea soon became somewhat controversial, as some of the initial observations could not be reproduced (Trebak *et al.*, 2003a; DeHaven *et al.*, 2009). Notably, the TRPC channels, as the mammalian TRP homologs are called, all appear to encode Ca^{2+} permeable, but relatively non-selective cation channels with properties clearly distinct from I_{crac} (Trebak *et al.*, 2003b; Beech *et al.*, 2004; Venkatachalam & Montell, 2007). The regulation of TRPC channels by phospholipase C, as well as their relationship to store-operated channel mechanisms, will be discussed in more detail in a subsequent section.

The molecular basis for store-operated calcium entry

The identification of the major molecular players in store-operated Ca^{2+} entry came almost a full twenty years after the articulation of the initial concept. In 2005, two laboratories, using limited RNAi screens, almost simultaneously identified STIM (stromal interacting molecule) as an essential component of store-operated Ca^{2+} entry (Roos *et al.*, 2005; Liou *et al.*, 2005). The following year, three laboratories, now using whole genome RNAi screens with *Drosophila* cells, identified a protein that had not previously been expressed or studied, and was at the time identified in the database as “hypothetical protein” (Feske *et al.*, 2006; Vig *et al.*, 2006b; Zhang *et al.*, 2006). The first group to publish the essential requirement of this protein named it Orai, who were “gatekeepers” in Greek mythology (Feske *et al.*, 2006). The specific roles played by the STIM and Orai proteins were soon thereafter revealed.

Mammals have two STIM proteins, STIM1 and STIM2, and three Orai proteins, Orai1, 2 and 3, all encoded by separate genes. The preponderance of experimental work has been carried out with STIM1 and Orai1. STIM1 resides in the endoplasmic reticulum and in the plasma membrane. There does not appear to be a role for the plasma membrane protein in store-operated Ca^{2+} entry (Mercer *et al.*, 2006), although it may play a role in another pathway involving arachidonic acid gated channels (Mignen *et al.*, 2007). Orai1 appears to reside almost exclusively in the plasma membrane. Overexpression of either these proteins alone has either a small or very little effect on store-operated entry. However, overexpression of the two together results in massive store-operated entry, and huge I_{crac} (Zhang *et al.*, 2006; Peinelt *et al.*, 2006; Mercer *et al.*, 2006). This result suggests that STIM1 and Orai1 are either sufficient to fully reconstitute store-operated Ca^{2+} entry, or if other stoichiometric components are required, they must already be present in considerable excess. A caveat with this conclusion is that it is based on overexpression; it is conceivable that at more modest levels of expression, signaling requires other participants whose roles become less obvious when STIM1 and Orai1 are present in huge excess.

STIM1 appears to function as the sensor of the Ca^{2+} level in the endoplasmic reticulum. STIM1 is a single pass membrane protein whose luminaly directed N-terminus contains one functional and one non-calcium binding EF-hand (Stathopulos *et al.*, 2008). When

endoplasmic reticulum Ca^{2+} levels fall, Ca^{2+} dissociates from the EF-hand, STIM1 molecules self associate (Liou *et al.*, 2007), migrate to near plasma membrane areas (Zhang *et al.*, 2005; Luik *et al.*, 2006), and activate the store-operated channels there composed of Orai subunits (Hewavitharana *et al.*, 2007; Prakriya, 2009). Perhaps the best evidence for the Ca^{2+} sensing function of the N-terminal hand is the finding that mutation of acidic residues within this domain results in constitutively active store-operated entry (Liou *et al.*, 2005).

It is interesting that a rather non-committal name was chosen for Orai; another group proposed the equally vague name “CRACM” for “CRAC Modulator” (Vig *et al.*, 2006b). This is likely due to the fact that Orai has no obvious sequence homology to any known ion channel, and there was uncertainty initially as to whether it was a part of the channel itself, or a regulator of the channel. The non-orthodox structure of Orai should not be too surprising however, recalling the unusually small single channel conductance estimated for I_{crac} . The result discussed above, whereby overexpression of the plasma membrane protein Orai1 with STIM1 reconstituted huge I_{crac} suggested that it might be the CRAC channel itself. The best evidence that this is so came from experiments examining the behavior of specific mutations of Orai1. Of particular interest is a glutamate at position 106 in the human sequence. Mutation of this residue to alanine resulted in complete loss of channel activity. More importantly, the more conservative mutation to aspartate resulted in a functional channel, but with significantly reduced selectivity for Ca^{2+} (Yeromin *et al.*, 2006; Vig *et al.*, 2006a; Yamashita *et al.*, 2007). This result provides strong evidence that Orai1 is a pore-forming subunit of the store-operated CRAC channel.

Physiological Functions of Store-operated Channels

The identification of key gene products constituting store-activated channels and their signaling mechanism has afforded an opportunity to learn more about their role in major physiological processes, especially those of significant clinical interest. In the initial studies leading to the identification of Orai, in addition to the RNAi screen in *Drosophila* cells, there was also information from mapping the locus of a mutation in Orai in humans that resulted in loss of store-operated entry and a severe immunodeficiency (Feske *et al.*, 2006). As a result, there has been considerable investigation of the role of Orai specifically, and of store-operated channels in general, in acquired immunity. Much of this work has been reviewed recently elsewhere, and will not be recapitulated here (Hogan *et al.*, 2010; Feske *et al.*, 2010; Oh-Hora, 2009; Baba & Kurosaki, 2009; Feske, 2007). Rather we choose to discuss three emerging and potentially clinically important areas: the proliferation and migration of vascular cells, thrombosis by the platelet and phagocytosis by innate immune cells.

Vascular Tissues

The abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) is the primary cause of atherosclerosis and restenosis. Calcium influx is known to be a critical regulator of the proliferation and contraction of VSMCs. With the identification of STIM1 and Orai1 as the molecular components of SOCE, a number of laboratories have investigated the contributions of STIM1 and Orai1 to the proliferation of VSMCs. In human coronary artery SMCs, STIM1 knock down suppressed cell growth, which could be due to a defect in SOCE-mediated phosphorylation of CREB protein (Takahashi *et al.*, 2007). Arterial smooth muscle cells (ASMCs) have the ability to change cell phenotype from quiescent and contractile to proliferative in response to physiological or pathological stimuli. Berra-Romani *et al.* (Berra-Romani *et al.*, 2008) demonstrated that while freshly isolated contractile SMCs showed only a small SOCE, cultured proliferative SMCs showed much larger SOCE (Berra-Romani *et al.*, 2008). This difference in the size of SOCE was associated with increased expression of STIM1 in cultured proliferative SMCs. Although Orai1 expression was only slightly upregulated in these cells, the expression of other Orai

isoforms, Orai2 and Orai3, and canonical transient receptor potential channels TRPC4 and TRPC5 were all significantly increased. Similar results were reported by Potier *et al.* (Potier *et al.*, 2009). Furthermore, Potier *et al.* demonstrated that knockdown of STIM1 and Orai1 suppressed the cell proliferation and migration of SMCs. Abnormal proliferation and migration of SMCs are the major causes of restenosis. The increase in STIM1 expression was also observed in *in vivo* experiments in which rat carotid arteries were injured by balloon angioplasty (Guo *et al.*, 2009). Strikingly, knockdown of STIM1 attenuated neointimal hyperplasia after balloon injury. Consistent with the *in vivo* data, cultured VSMCs infected with adenovirus coding shRNA against STIM1 showed less proliferation and migration compared to control cells. Cell cycle analysis demonstrated that STIM1 deficient cells were mostly arrested at the G₀/G₁ phase, indicating that STIM1 is critical for progression into S phase in proliferating VSMCs. This cell cycle arrest at G₀/G₁ was possibly caused by an increased expression of cyclin-dependent kinase inhibitor p21 and decreased phosphorylation of retinoblastoma protein (Guo *et al.*, 2009). In this vascular injury model, the actual stimuli inducing VSMC proliferation and migration were unknown. Bissailon *et al.* (Bissailon *et al.*, 2010) focused on platelet-derived growth factor (PDGF) which plays a prominent role in migration of VSMCs into the neointima following acute vessel injury and in the lesions of atherosclerosis (Bissailon *et al.*, 2010; Raines, 2004). In fact, PDGF-induced cell migration was strongly diminished in either STIM1- or Orai1-knockdown VSMCs. Interestingly, knock down of STIM2, Orai2 or Orai3 did not suppress PDGF-induced VSMC migration.

Abnormal proliferation and migration of VSMCs are not the sole cause of atherosclerosis and restenosis; proliferation and migration of vascular endothelial cells (VECs) may contribute as well. Abdullaev *et al.* (Abdullaev *et al.*, 2008) demonstrated that the suppression of STIM1 or Orai1 almost completely abrogated SOCE in VECs. STIM1 and Orai1 knockdown also suppressed the normal growth of VECs. Interestingly, the defect of proliferation due to loss of SOCE was accompanied by cell cycle arrest at S and G₂/M phase (Abdullaev *et al.*, 2008). Thus, the effect on VECs differs from that for VSMCs which were arrested in the G₀/G₁ phase. This would imply that the specific steps in cell cycle regulation that are impacted by SOCE may differ in the two cell types.

Platelets

Platelets are anucleated, non-excitabile blood cells circulating in the blood. When the vascular wall is damaged, platelets are recruited and activated and aggregate to form clots at the site, thus playing a critical role in hemostasis to prevent excess blood loss from vascular injury. However, excess building of blood clots can lead to thrombosis and eventually to myocardial infarction or ischemic stroke. An increase in intracellular Ca²⁺ concentration is one of first signals in platelet activation. A critical contribution of STIM1 platelet function was first described in a study by Grosse *et al.* (Grosse *et al.*, 2007), in which a mouse line bearing a mutation of the EF hand motif in the STIM1 N-terminus was identified and characterized (Grosse *et al.*, 2007). This mouse line (Saxcoburggotski; STIM1^{sax/+}) was originally established with dominant inheritance of elevated mean platelet volume and reduced platelet counts in comparison to WT controls. The homozygote of this mouse line (STIM1^{sax/sax}) was embryonically lethal and featured severe hemorrhages in their embryonic body. Despite the reduced number of platelets, the mutation in STIM1 does not affect megakaryocyte differentiation in STIM1^{sax/+} mice. The authors observed an increased basal Ca²⁺ concentration in the platelets from STIM1^{sax/+}, which suggests preactivation of the STIM1^{sax/+} platelets leading to a decreased life span. Interestingly, despite elevated basal Ca²⁺ concentrations, thapsigargin induced Ca²⁺ responses were significantly suppressed in STIM1^{sax/+} platelets. Furthermore, although Ca²⁺ responses from the G_q protein-coupled agonist, thrombin, were largely unaffected in STIM1^{sax/+} platelets, ITAM

(immunoreceptor tyrosine-based activation motif) receptor-mediated Ca^{2+} responses were largely inhibited. This defect in Ca^{2+} responses is presumable responsible for the defect in collagen-dependent platelet aggregation and thrombus formation in $\text{STIM1}^{\text{sax/+}}$ mice.

Later, Varga-Szabo et al. (Varga-Szabo *et al.*, 2008) generated STIM1 -null mice by using gene trapping. The majority of homozygote of STIM1 -null mice again showed either embryonic lethality or very low survival rate. In those surviving mice, megakaryopoiesis or platelet production was normal, and there was no apparent difference in coagulation. In response to thapsigargin, platelets from $\text{STIM1}^{-/-}$ mice showed reduction of both Ca^{2+} release from intracellular stores and subsequent Ca^{2+} influx, indicating that STIM1 serves to maintain intracellular Ca^{2+} stores, presumably through SOCE, in mouse platelets. Because of the early mortality of the $\text{STIM1}^{-/-}$ mice, Varga-Szabo et al. (Varga-Szabo *et al.*, 2008) generated chimeric $\text{STIM1}^{-/-}$ mice by transplanting $\text{STIM1}^{-/-}$ bone marrow into lethally irradiated wild-type mice. Consistent with the defect of SOCE, the Ca^{2+} responses to both G_q protein-coupled receptor agonists and ITAM receptor agonists were severely reduced in $\text{STIM1}^{-/-}$ platelets. However, platelet aggregation and activation were only suppressed in response to an ITAM receptor agonist. The authors also demonstrated that $\text{STIM1}^{-/-}$ chimeric mice showed less thrombosis in a model of thrombosis induced by arterial and arteriole injury. Furthermore, in an ischemic brain infarction model, $\text{STIM1}^{-/-}$ chimeric mice were significantly resistant to ischemia-induced brain damage (Varga-Szabo *et al.*, 2008). This same group also developed $\text{Orai1}^{-/-}$ chimeric mice and obtained results similar to those with the $\text{STIM1}^{-/-}$ chimeras (Braun *et al.*, 2009b). These studies indicate that STIM1 and Orai1 -mediated SOCE is important for platelet activation and subsequent thrombosis. In addition, these reports suggest a minor contribution of STIM1 and Orai1 for primary hemostasis induced by mechanical injury. However, contradictory results have also been reported. Platelets from mice expressing the SCID mutation in Orai1 (R93W) showed significant reduction of platelet activation in response to both G_q protein-coupled and ITAM receptor agonists (Bergmeier *et al.*, 2009). In addition, Galan et al. (Galan *et al.*, 2009) reported that STIM1 and Orai1 play critical roles in both thrombin- and ADP-induced platelet aggregation in human platelets, in which STIM1 and Orai1 function was suppressed by the introduction into the platelets of antibodies against both proteins (Galan *et al.*, 2009).

Phagocytes

Phagocytosis is a process of innate immunity involving the clearance of pathogens, apoptotic cells and cellular and foreign debris. The first step of the multistep process of phagocytosis is recognition of ligand on the particle surface by receptors such as the $\text{Fc}\gamma$ receptors ($\text{Fc}\gamma\text{R}$) which evokes a PLC-mediated Ca^{2+} increase. This Ca^{2+} increase regulates many but not all phagocytic processes. It has long been known that chelation of intracellular Ca^{2+} or removal of extracellular Ca^{2+} inhibits the maturation of phagosome, specifically, ROS production, acidification of the phagosome and fusion of endosomal and secretory vesicles (reviewed in (Nunes & Demareux, 2010).

The discovery of STIM and Orai proteins shed new light on the dependence of each Ca^{2+} -dependent step in phagocytosis on different Ca^{2+} sources.

Steinckwich et al. (Steinckwich *et al.*, 2011) recently reported that STIM1 and Orai1 -mediated Ca^{2+} influx is critical for ROS production in phagosomes, while Ca^{2+} release is important for the first particle internalization step in the human promyelocytic cell line HL-60 differentiated into neutrophil-like cells (dHL-60). The authors further identified S100A8 and S100A9 small Ca^{2+} binding proteins as the mediators of STIM1 - and Orai1 -mediated SOCE and ROS production. S100A8 and S100A9 proteins are localized in the perinuclear region and in the cytoplasm in resting cells but relocate to phagosomes when they form particle internalization (Steinckwich *et al.*, 2011). This same group previously had

reported that knock-down of STIM1 and STIM2 in dHL-60 cells suppressed formyl peptide-induced ROS production (Brechard *et al.*, 2009). STIM1 but not STIM2 abrogation impaired thapsigargin-induced SOCE and also inhibited Ca^{2+} responses to the more physiological stimulus, the formylated peptide fMLF. Consistent with the findings for Ca^{2+} entry upon activation of fMLF treatment, STIM1 knockdown, but not STIM2 knockdown, caused an eventual suppression of hydrogen peroxide production mediated by one of the NADPH oxidases. These data indicate that SOCE mediated by STIM1 and Orai1 functions as a trigger for ROS production regardless of the upstream receptor type; Fc γ R is an ITAM receptor and fMLF receptor is a G protein-coupled receptor. The defect in phagocytosis due to STIM1 deficiency was also observed in peritoneal macrophages from STIM1 $^{-/-}$ chimeric mice produced by bone marrow transplantation (Braun *et al.*, 2009a). The STIM1 $^{-/-}$ macrophages showed diminished intracellular Ca^{2+} mobilization in response to either thapsigargin or an Fc γ R agonist. Consistent with the defect in Fc γ R-activated Ca^{2+} responses, STIM1 $^{-/-}$ macrophages failed to ingest IgG-coated red blood cells. Phagocytosis is also important for clearance of apoptotic cells. Gronski *et al.* (Gronski *et al.*, 2009) reported that STIM1 knockdown in *C. elegans* suppressed phagocytosis of apoptotic cells, possibly due to a defect in actin cytoskeleton rearrangement during phagocytotic cup formation. These studies suggest that STIM1 and Orai1-mediated SOCE may contribute to the regulation of phagocytosis at multiple steps, including phagocyte formation and maturation.

TRPC Channels

In addition to the small family of three store-operated Orai channels, another somewhat larger family of ion channels are also linked to phospholipase C, although not exclusively to Ca^{2+} store depletion. These are a sub-family of the larger family of TRP channels, called TRPC for canonical TRPs, because they are most closely related in both structure and function to the founding *Drosophila* photoreceptor channel, TRP (Montell *et al.*, 2002). In mammals, there are 7 TRPC channels, designated TRPC1 through TRPC7, although TRPC2 is a pseudogene in humans. As is the case for their *Drosophila* counterparts, all appear to be activated downstream of phospholipase C. There may be two fundamental phospholipase C-mediated activation mechanisms for TRPC channels. The most widely accepted mechanism, and for which there is currently no debate, is a mechanism that depends upon the products of phospholipase C in some manner, but not upon the depletion of intracellular Ca^{2+} stores. This was most clearly demonstrated for *Drosophila* TRP, which was efficiently activated by light (through phospholipase C) in flies lacking IP₃ receptors (Acharya *et al.*, 1997). Trebak *et al.* (Trebak *et al.*, 2003a) investigated the activation of mammalian TRPC3 channels ectopically expressed in kidney cell line (HEK293). In this system, TRPC3 channels were efficiently activated when phospholipase C was activated through muscarinic cholinergic receptors, but not when Ca^{2+} stores were depleted with thapsigargin. Injection of IP₃ into the cells resulted in rapid and substantial release of Ca^{2+} stores, but no activation of TRPC3. Finally, blockade of IP₃ receptors by intracellular application of heparin completely prevented the discharge of intracellular stores by a muscarinic receptor agonist, but did not prevent the activation TRPC3. Similar studies have provided similar evidence for other members of the TRPC family, with the possible exception of TRPC1, discussed in more detail below (Trebak *et al.*, 2007).

What then is the precise signal originating from phospholipase C activity that is responsible for TRPC channel activation? This question has not as yet been definitively answered. Since IP₃ and *vide infra* store depletion seem not to be involved, the logical alternative is the other reaction product diacylglycerol. Indeed, Hofmann *et al.* (Hofmann *et al.*, 1999) reported that TRPC6 channels could be directly activated by diacylglycerol, either by application of membrane permeable diacylglycerol analogs to intact cells, or by application of

diacylglycerols to channels in excised patches. TRPC3, 6 and 7 all appear to behave similarly: they can all be activated independently of phospholipase C activity by diacylglycerols (Vazquez *et al.*, 2004a). This does not appear to involve protein kinase C, since activators of protein kinase C are potent inhibitors of TRPC channels (Trebak *et al.*, 2005; Venkatachalam *et al.*, 2003). Nonetheless, it is questionable as to whether the effect of diacylglycerol on the channels is direct. First, Smyth *et al.* (Smyth *et al.*, 2005) showed that tyrosine kinase-coupled growth factors could increase the surface expression of TRPC3 channels; however, the newly expressed channels could not be activated by synthetic diacylglycerols, while those already present could. Second, the signaling tyrosine kinase, src, was shown to be essential for diacylglycerol activation of TRPC3, but was not required for the channel's constitutive activity (Vazquez *et al.*, 2004b). Third, in contrast to the earlier report for TRPC6, Lemonnier *et al.* (Lemonnier *et al.*, 2008) observed activation of single TRPC7 channels by synthetic diacylglycerols in cell attached mode, but this activation was lost upon excision of the patch.

TRP channels, including TRPC channels, appear to be comprised of a tetrameric assembly of TRPC molecules (Abramowitz & Birnbaumer, 2008). Heteromeric assemblies of TRPC subunits can occur, at least experimentally, although perhaps with certain restrictions. Hofmann *et al.* (Hofmann *et al.*, 2002) reported that, in keeping with their apparent common mode of activation, TRPC3, 6 and 7 readily combine to form heteromeric channels, but these TRPCs cannot combine with TRPC1, 4 or 5. Likewise, TRPC1, 4 and 5 can combine with one another, but not readily with TRPC3, 6 or 7. In one study combinations of TRPC1 with TRPC5 resulted in channels with distinct properties from homotetramers of TRPC5 (Strübing *et al.*, 2001). Note however, that other labs have sometimes reported combinations outside of these rules, based on co-immunoprecipitation

There has been little work on TRPC2 because it is not a functional gene in humans; it may also be activated by diacylglycerol (Lucas *et al.*, 2003). TRPC1 is probably a special case, discussed below. TRPC4 and 5 are both clearly activated downstream of phospholipase C, but not by diacylglycerol.

The activation mechanism for TRPC4 and 5 has been difficult to resolve. It is rather clear that phospholipase C is important for their activation, as receptor activation of these TRPCs is blocked by a phospholipase C inhibitor. Schaefer *et al.* (Schaefer *et al.*, 2000) demonstrated that TRPC4 and 5 (1) can be activated by either PLC β or PLC γ -coupled receptors, but not by store depletion; and (2) activation was blocked by the PLC antagonist U73122. However, neither IP₃ nor diacylglycerol activated these channels. Subsequently, other reports have provided evidence that Ca²⁺ (Blair *et al.*, 2009; Gross *et al.*, 2009) can strongly augment TRPC5 currents. Also, G α i subunits have been reported to activate the channels (Jeon *et al.*, 2008). However, neither of these mechanisms can explain the general sensitivity of TRPC4 and 5 to PLC activation, whether through PLC β or PLC γ . Also, the failure of the classical activator of SOC channels, thapsigargin, when GPCR activation works well, argues that Ca²⁺ cannot be the initiator of the activation mechanism.

One suggested mechanism for TRPC5 activation is the depletion of plasma membrane PIP₂ (Trebak *et al.*, 2009). Generally, ion channels are activated or potentiated by polyphosphoinositides (Suh & Hille, 2005; Hilgemann *et al.*, 2001) and this appears to be the case for TRP channels as well (Voets & Nilius, 2007; Rohacs & Nilius, 2007). This is also the case for the TRPC channels TRPC3, 6, 7 and 5 when studied in excised patch mode (Trebak *et al.*, 2009; Lemonnier *et al.*, 2008), despite the fact that in intact cells, these TRPC channels are turned on subsequent to PLC activation. One tool used in the study of PIP₂ regulated channels is a small group of pharmacological inhibitors of the enzyme that phosphorylates PI to PIP, PI 4-kinase. The two most commonly used, wortmannin and

LY294002, are potent inhibitors of the PI 3-kinase, but at a 10-fold higher concentration, they block PI 4-kinase (Linseman *et al.*, 1999; Linseman *et al.*, 1998). When applied in this concentration to cells expressing TRPC5, the compounds activated TRPC5-dependent $[Ca^{2+}]_i$ signals and TRPC5 currents (Trebak *et al.*, 2009). Under similar experimental conditions, TRPC3 channels were not activated. Yet, in excised patches, TRPC5 single channels were strongly activated by addition of PIP2 to the patch, and to a greater extent than that seen with receptor activation in intact cells. To explain these contradictory findings, Trebak *et al.* (Trebak *et al.*, 2009) proposed two distinct functions of PIP2. PIP2 may associate directly with the channels and may be required for their activity, as appears to be the case for many other ion channels. In addition, PIP2 may have a signaling function which involves its interaction with a regulatory molecule. PIP2 binding to this regulator causes it to bind to the channels and inhibit their activity. Activation of PLC would specifically deplete the pool of PIP2 involved in braking TRPC5 activity leading to channel activation. In excised patches, loss of both pools of PIP2 results in a channel lacking the negative regulator, but also lacking the PIP2 having a necessary co-factor function for channel activity. Addition of PIP2 in this condition would be expected to produce strong channel activation, as was observed experimentally.

Interestingly, a similar difficulty exists for the founding member of the TRP superfamily, the photoreceptor TRP channel in *Drosophila* (Hardie, 2003). As for mammalian TRPCs, which are most closely related to the *Drosophila* channel, *Drosophila* TRP is absolutely dependent upon PLC activity. Experimental evidence suggests that changes in the lipid composition of the plasma membrane, as a result of PLC activation, play at least a positive modulatory role in the activation of *Drosophila* TRP. An intriguing hypothesis recently put forth by Huang *et al.* (Huang *et al.*, 2010) is that TRP may be activated by the protons released in the hydrolysis reaction of PLC. As to whether this might play a role in the activation of mammalian TRPCs requires further investigation.

Cross-talk between TRPCs and SOC

As discussed above, there has been continuing controversy over the question of whether or not TRPs, especially TRPCs, can function as store-operated channels. The early reports of expressed TRPCs being activated by Ca^{2+} store depletion (Zhu *et al.*, 1996; Kiselyov *et al.*, 1998; Liu *et al.*, 2000; Xu & Beech, 2001) were not subsequently reproduced in other laboratories (Schaefer *et al.*, 2000; Trebak *et al.*, 2003a; DeHaven *et al.*, 2009; Potier *et al.*, 2009). In most cases it is unclear as to what are the discrepancies between and among the many published studies (and only a few representative ones are cited here). With the discovery of Orai, the molecular nature of the channel underlying the classical store-operated current, I_{crac} , became quite clear. It has been suggested by one group that TRPC channels might complex with Orai in some way to form CRAC channels (Liao *et al.*, 2009; Liao *et al.*, 2008; Liao *et al.*, 2007). Some clarification may be emerging, however, with regard to one of the TRPCs: TRPC1. Knockdown or overexpression of TRPC1 appears to abrogate or augment, respectively, store-operated Ca^{2+} entry in certain experimental circumstances (Liu *et al.*, 2000; Ambudkar & Ong, 2007). In a salivary gland cell line, Ca^{2+} store depletion gives rise to a $[Ca^{2+}]_i$ signal and a current that appears less selective than I_{crac} , termed I_{soc} (Liu *et al.*, 2003). Knockdown of TRPC1 reduces I_{soc} (Liu *et al.*, 2007); however, loss of Orai1 causes complete loss of this current (Cheng *et al.*, 2008). In a recent study, Cheng *et al.* (Cheng *et al.*, 2011) describe a series of experiments that resolve the relative roles of TRPC1 and Orai1 in the generation of I_{soc} . First, whereas knockdown of Orai1 caused a complete loss of the current, knockdown of TRPC1 resulted in a loss of only a part of the total current; importantly, the residual current was now indistinguishable from I_{crac} . Thus, they conclude that I_{soc} is actually comprised of two distinct currents, I_{crac} through Orai1 channels, and a relatively linear current through TRPC1 channels. However,

Ca²⁺ entry through the store-operated channels is absolutely required for any channel activation. Their findings indicate that Ca²⁺ entry through CRAC channels signals translocation of TRPC1 to the plasma membrane. This would then indicate that TRPC1 is not a store-operated channel, but is in fact a Ca²⁺-activated channel or sorts. However, they also find, as have others, that the activation of TRPC1 requires a direct interaction between STIM1 and TRPC1, in addition to the role of STIM1 in activating Ca²⁺ entry through Orai1 channels. Thus, the issue of whether or not TRPC1 is a store-operated channel becomes semantic. Store depletion is *necessary* for activation of TRPC1, but store depletion is not *sufficient* in the absence of a primary Ca²⁺ entry through Orai1 channels.

This situation helps explain at least some of the findings in one conflicting report. DeHaven *et al.* (DeHaven *et al.*, 2009). These authors examined the ability of store-depletion with thapsigargin to activate a number of TRPC channels, including TRPC1, when ectopically expressed in HEK293 cells. In experiments utilizing measurements of cytoplasmic Ca²⁺, TRPC3, 6, 5 and 7 could all be activated by the PLC pathway, but not by store depletion. TRPC1 could not be activated by either means. Because the HEK293 cells contain endogenous Orai1 channels, in order to observe [Ca²⁺]_i signals from the TRPC channels that are not contaminated with [Ca²⁺]_i signals from the endogenous Orai channels, the Orai channels were specifically blocked with low concentrations of Gd³⁺ (Broad *et al.*, 1999; Trebak *et al.*, 2003a). However, based on the recent findings of Cheng *et al.* (Cheng *et al.*, 2011), TRPC1 cannot be activated without concomitant activation of Orai channels. This could readily explain why no activation of TRPC1 was observed by DeHaven *et al.* with either of the two means of stimulation. It might also explain why the other TRPCs in the study were activated through the receptor pathway but not by the strictly store-operated pathway (i.e., with thapsigargin). If so, this would mean that TRPCs, other than TRPC1, can function in two modes: they can be activated by PLC-derived signals independently of Ca²⁺ store depletion, and/or they can be activated in a coordinated way together with store-operated Orai channels. Note that the polybasic sequence in STIM1 needed for interaction with TRPC1 is found in most species, but it is notable absent in *Drosophila*.

Conclusions

A variety of signaling pathways are linked to the activation of phospholipase C, including two mechanisms of plasma membrane Ca²⁺ influx, discussed in this review. A summary of the two mechanisms and their link to phospholipase C is given in Figure 1. The knowledge of these key pathways has grown to the point where we may soon expect translation of the basic science to improved therapies for a number of human diseases.

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Phospholipase C-activated Calcium Permeable Ion Channels

Figure.

Agonist (Ag) activation of a receptor (R) leads to activation of phospholipase C (PLC), in some instances through an intermediate G protein (G) resulting in the release of IP₃, formation of diacylglycerol (DAG) and a decrease in PIP₂. IP₃ activates an IP₃ receptor (IP₃R) on the endoplasmic reticulum resulting in release of stored Ca²⁺. The drop in endoplasmic reticulum Ca²⁺ activates a Ca²⁺ sensor, STIM1, that redistributes within the endoplasmic reticulum to sites where it can interact with and activate the store-operated Orai1 Ca²⁺-selective channels. Another class of less selective, Ca²⁺-permeable channels, the TRPCs, are activated by PLC, most likely by changes in membrane composition of DAG and PIP₂. Some TRPC channels are inhibited by protein kinase C and/or require the tyrosine kinase, src. Not shown in this figure is a presumed complex interaction between the pathways involving the translocation of TRPC channels to the membrane in response to Ca²⁺ entering through Orai1 channels (see (Cheng *et al.*, 2011)).