Calcium Entry Mechanisms in Salivary Glands

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Abstract

The organic and inorganic milieu of the oral cavity is regulated in large part through the secretions of salivary glands. These secretions are activated by a number of cellular signaling mechanisms, a major one being the cellular Ca^{2+} signaling system. Activation of phospholipase C by G-protein-coupled receptors results in release of intracellular Ca^{2+} and activation of Ca^{2+} channels in the plasma membrane. The intracellular release of Ca^{2+} is signaled by the second messenger, inositol 1,4,5-trisphosphate. Ca^{2+} entry involves signaling from depleted intracellular stores to plasma membrane Ca^{2+} channels, a process referred to as *capacitative calcium entry* or *store-operated calcium entry*. The electrophysiological current associated with capacitative calcium entry is the calcium-release-activated calcium current, or I_{crac} . In the twenty years since the inception of the concept of capacitative calcium entry, a variety of activation mechanisms have been proposed, and there has been considerable interest in the possibility of transient receptor potential channels functioning as store-operated channels. However, in the past two years, two major players in both the signaling and permeation mechanisms for store-operated channels have been discovered: Stim1 (and possibly Stim2) and the Orai proteins. Activation of store-operated channels involves an endoplasmic reticulum Ca^{2+} sensor called Stim1. Stim1 acts by redistributing within a small component of the endoplasmic reticulum, approaching the plasma membrane, but does not appear to translocate into the plasma membrane. Stim1, either directly or indirectly, signals to plasma membrane Orai proteins which constitute pore-forming subunits of store-operated channels.

Keywords :

salivary glands, signal transduction, calcium signaling, calcium entry, calcium channels

Introduction

The normal physiological milieu of the oral cavity is influenced by a number of environmental factors, both internal and external. Among the most important internal factors is the amount and composition of saliva. Saliva is well documented to be important for maintaining oral health, and especially dental health (1-5). Thus, understanding abnormalities in salivary gland function, for example in Sjogren's syndrome (3), is key to efforts to circumvent these abnormalities and restore normal salivary function.

The process of salivation is a complex one. While there is a certain constant, constitutive flow of secretion into the oral cavity, the majority of saliva is

generated in response to complex reflex neural mechanisms that greatly increase the rate of salivary flow. These neural mechanisms culminate in the release of neurotransmitters primarily onto salivary acinar cells. Activation of the neurotransmitter receptors on the acinar cell surface activates basic signaling mechanisms which set into play complex cellular responses that culminate in the generation of transepithelial fluid flow, and the exocytotic discharge of macromolecules (6, 7).

Two basic signaling pathways regulate salivary secretion. Both involve the activation of surface membrane receptors linked to guanine nucleotide dependent transducers, or G-proteins. The G-pro-

teins activate effector enzymes that produce specific second messenger molecules. One, linked primarily to β -adrenergic receptors activated by sympathetic input, acts through the second messenger cyclic AMP. Cyclic AMP activates a specific kinase leading to phosphorylation of proteins that regulate both exocytotic secretion and fluid secretion (6, 8-11). The second major pathway involves activation of phospholipase C and generation of second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (12).

Activation of phospholipase C

Salivary glands contain a number of different G-protein coupled receptors that link to the activation of phospholipase C, including cholinergic muscarinic, α -1 adrenergic, substance P, and P2Y purinergic, to name a few. These receptors act through G-proteins of the $G_{q/11}$ class to activate phospholipase C (PLC), primarily $\text{PLC}\beta$. PLC cleaves plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the Ca^{2+} -signaling messenger, IP_3 , and the protein kinase C-activating messenger, DAG (13). These two pathways interact in the regulation of protein secretion which appears to operate optimally with a combined Ca^{2+} and protein kinase C signal (14). Ca^{2+} also serves as a signal for regulating membrane ion channels leading to fluid secretion; the role of protein kinase C in this pathway is less clear.

Two phases of Ca^{2+} signaling

Activation of PLC in salivary gland cells results in production of IP_3 and a subsequent rise in the concentration of Ca^{2+} in the cytoplasm, generally referred to as a Ca^{2+} signal. In the initial studies of Ca^{2+} signaling in salivary gland preparations *in vitro*, indirect measures of cytoplasmic Ca^{2+} were employed, for example, the activity of Ca^{2+} -activated K^+ channels by measuring the rate of $^{86}\text{Rb}^+$ efflux (Fig. 1). From these earliest measurements, it soon became obvious that the Ca^{2+} signal was comprised of two distinct phases: an initial phase that was independent of extracellular Ca^{2+} and involved

release of Ca^{2+} from the cell's interior, and a second, more sustained phase that involved the activation of Ca^{2+} entry into the cell across the plasma membrane (15, 16) (Fig. 1). The mechanism underlying the first, Ca^{2+} -release phase, is attributed to the action of the PLC product, IP_3 (13, 17). IP_3 releases Ca^{2+} from the endoplasmic reticulum, or a specialized component of it, by binding to and activating specific IP_3 receptor/ion channels in the endoplasmic reticulum membrane (18). Early studies indicated that the pools of Ca^{2+} regulated by different PLC-coupled receptors were coincident, as expected if regulated by a common diffusible messenger (16). With lower, more physiological concentrations of agonists, this release often occurs in the form of repetitive transient rises referred to as Ca^{2+} oscillations (19, 20). Oscillations can take either of two forms: baseline spikes or sinusoidal oscillations (21). In the parotid gland, the predominant form is sinusoidal (22, 23). These oscillations likely result from fluctuations in PLC activity due to negative feedback from protein kinase C (23).

Fig. 1. Pattern of Ca^{2+} signaling in parotid cells revealed by flux of $^{86}\text{Rb}^+$. The rate of efflux of $^{86}\text{Rb}^+$ is monitored as an index of the activity of Ca^{2+} -activated K^+ channels. Addition of an agonist for a phospholipase C-coupled receptor leads to an increase in $[\text{Ca}^{2+}]_i$ ($^{86}\text{Rb}^+$ flux) which is sustained in the presence, but transient in the absence of external Ca^{2+} . These two phases represent Ca^{2+} signaling due to release (initial transient) and Ca^{2+} entry across the plasma membrane (sustained component).

Accompanying or immediately following the Ca^{2+} release phase of the response is a phase of Ca^{2+} signaling involving Ca^{2+} entry into the cell through channels in the plasma membrane (16). Consistent with the idea of a common pool of Ca^{2+} controlled by different PLC-coupled receptors, it was also apparent that a single population of Ca^{2+} channels were regulated by different receptor types (16). While the molecular basis for the intracellular release of Ca^{2+} was quickly solved (IP_3 and IP_3 receptors), the molecular basis for the coupled activation of Ca^{2+} entry into salivary gland cells, and other cells in which PLC played a predominant role was not understood until very recently. This story will be the main focus of the remainder of this review.

Capacitutive Calcium Entry

Experiments carried out in parotid gland cells provided the first evidence for a mechanism called *capacitative calcium entry* or *store-operated calcium entry* (16, 24-26). The term “capacitative” calcium entry was originally meant to imply this continuous loading and discharge of a Ca^{2+} store, much as in an electrical circuit, charge must load a capacitor before current can flow through it. In my own mind, this obligatory linkage of release to entry came about from experiments showing reloading of stores through Ca^{2+} entry in parotid and lacrimal acinar cells in two studies published in 1977 (16) and 1978 (27), the latter being the first report to demonstrate refilling of Ca^{2+} stores independently of receptor activation. These two papers concluded that the process of Ca^{2+} entry and release were essentially one and the same, and that entry proceeded through release sites to the cytoplasm. It was not clear where the release sites were at that time, and for the sake of simplicity, it was assumed Ca^{2+} was bound to the plasma membrane itself (Fig. 2).

The precise pathway by which Ca^{2+} reloaded the internal stores was not initially clear; in 1978 I pointed out that no activation of K^+ ($^{86}\text{Rb}^+$) efflux (an indicator of $[\text{Ca}^{2+}]_i$ changes) was observed (24, 27), suggesting either a direct route, or possibly one compartmentalized or protected from the bulk of

Fig. 2. *Primitive version of capacitutive calcium entry.* The intracellular Ca^{2+} store was envisioned as bound to the plasma membrane, occluding the Ca^{2+} channel. release of the bound Ca^{2+} was associated with gating of the Ca^{2+} channel. The figure is similar to one first presented in (16).

cytoplasm. This of course turned out to be wrong. With hindsight, it is now clear that the failure of $[\text{Ca}^{2+}]_i$ to rise significantly during refilling is simply due to the buffering speed of endoplasmic reticulum matching or exceeding the rate of Ca^{2+} entry, and possibly (see below) close apposition of plasma membrane channels with endoplasmic reticulum uptake sites. In fact, in some cell types, when the rate of re-filling is pushed to the maximum, transient elevation of $[\text{Ca}^{2+}]_i$ is observed (25). However, the clearest evidence that Ca^{2+} must enter the cytoplasm directly came from experiments with the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase inhibitor, thapsigargin (26), and from the subsequent demonstration of a store-operated Ca^{2+} current by Hoth and Penner (28).

Action of Thapsigargin

Experiments with the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor, thapsigargin, in parotid cells provided an important proof for the concept of capacitutive calcium entry. The ability to activate Ca^{2+} entry simply by blocking the intracellular pumps responsible for its sequestration

led to a number of important conclusions (26). First, it was possible to show that entry could be fully activated with no discernible activation of phospholipase C. Second, additivity experiments showed that, at least in the parotid cells that were initially studied, there was no additional pathway activated through phospholipase C-linked muscarinic receptors. Third, the fact that thapsigargin activated Ca^{2+} entry to the same extent as phospholipase C activation, but without increasing IP_3 demonstrated that passage through the endoplasmic reticulum was not required for access to the cytoplasm; i.e., the permeability of the endoplasmic reticulum membrane to Ca^{2+} was not a determinant of the rate at which Ca^{2+} entered the cytoplasm. This and other arguments favoring a transmembrane flux of Ca^{2+} rather than a transit through intracellular stores were summarized in an earlier review (29).

But, perhaps the most significant outcome of the discovery of thapsigargin and other SERCA-inhibiting drugs (Fig. 3) was that it provided a precise functional and pharmacological definition of

capacitative entry. Notably, the ability of thapsigargin to specifically activate capacitative calcium entry, while minimizing the roles of other upstream players in the pathway (receptors, G-proteins, phospholipase C, etc.) formed the basis for the high throughput assays that led to the discoveries of two significant molecular players in this pathway, discussed in a later section, Stim and Orai.

I_{crac}

That Ca^{2+} entered the cytoplasm by directly traversing the plasma membrane was unequivocally established by the demonstration of a store-operated or capacitative calcium entry transmembrane current (28). Hoth and Penner (28, 30) named this current Calcium-Release-Activated Ca^{2+} current, or I_{crac} , and provided a thorough biophysical characterization of this very small but highly Ca^{2+} -selective current. I_{crac} shared some properties with previously studied Ca^{2+} -selective voltage-activated currents. For example, I_{crac} demonstrated an anomalous mole fraction effect; removing external Ca^{2+} led to loss of current, unless all external divalent cations were removed with chelators, in which case the currents became permeable to monovalent cations, and were actually larger than when conducting Ca^{2+} . However, I_{crac} differed from other known currents in one significant way: the single channel conductance was too small to be measured directly, and was subsequently estimated indirectly by noise analysis by Zweifach and Lewis (31) to be of the order of 24 fS (in isotonic Ca^{2+}). This finding suggests that the molecular structure of CRAC channels and the other known Ca^{2+} channels might be very different (which in fact has turned out to be the case). In addition, I_{crac} showed unusual regulation by Ca^{2+} ; intracellular Ca^{2+} inhibited the channels by multiple mechanisms (30, 32), while extracellular Ca^{2+} potentiated channel function (33). These and a number of other findings provided a rigorous fingerprint for the CRAC channel that eventually aided in its correct molecular identification.

SERCA Inhibitors

Fig. 3. Structures of commonly employed inhibitors of sarco-plasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA).

Numerous activation mechanisms are proposed

Several reviews have summarized the ideas that have come in and out of vogue in the last twenty years regarding the activation mechanism for capacitative calcium entry (34-40). Three fundamental mechanisms have been proposed for transmitting the signal from intracellular stores to the plasma membrane: (i) a diffusible message, (ii) conformational coupling (35), and (iii) vesicle secretion (41).

The idea of a diffusible message for capacitative calcium entry was proposed when it was realized that no special path for calcium entry through the endoplasmic reticulum existed, and that the calcium entered the cell directly across the plasma membrane into the cytoplasm (29). Subsequently, a considerable number of publications have argued for a diffusible signal coupling depletion of intracellular Ca^{2+} stores to calcium entry, and some evidence, albeit more controversial, for the involvement of specific mediators. Some have suggested involvement of cyclic GMP (42, 43, 43), others, arachidonic acid (or one of its metabolites) (44-46); however, it now seems likely that these mediators act on channels distinct from the store-operated ones (47, 48). The one candidate for a diffusible signal for store-operated channels that has withstood the test of time is one whose structure is as yet not known: a Ca^{2+} entry-activating principle partially isolated from store-depleted cells called CIF (for Calcium Influx Factor) (39, 49, 50). The first experimental evidence for such a messenger was provided by Randriamampita and Tsien (49). They described an activity in extracts of stimulated Jurkat T cells that increased Ca^{2+} entry in several cell types. This activity they termed "CIF" for Calcium Influx Factor. Further fractionation and purification of cell extracts produced an activity, probably different from the first CIF described by Randriamampita and Tsien, that potently activated Ca^{2+} influx when injected into *Xenopus* oocytes (51) or when applied to patches containing excised, store-operated channels (52, 52, 53, 53, 54). The most recently published data on CIF action present a compelling case for such a molecule, and suggest a mechanism whereby CIF acts to regulate a Ca^{2+} -

independent phospholipase A_2 which in turn generates a channel-activating lysophospholipid (53-56). The role, if any, of CIF in the newly discovered Stim1-dependent activation mechanism, discussed below, awaits additional experimental work.

(54) Irvine (57) (see also (35)) proposed a conformational coupling model based on the known interaction between plasma membrane dihydropyridine receptors (voltage-dependent calcium channels) and intracellular ryanodine calcium release channels in skeletal muscle. In the phospholipase C signaling system, endoplasmic reticulum IP_3 receptors were proposed to interact directly with plasma membrane capacitative calcium entry channels. In the case of skeletal muscle, information flows from the t-tubule membrane to the sarcoplasmic reticulum; in the case of the IP_3 receptor and capacitative calcium entry, a fall in luminal Ca^{2+} in the endoplasmic reticulum would induce a conformational change in the IP_3 receptor, and this would be conveyed directly to the plasma membrane channel via a protein-protein interaction. This was an intriguing idea, but there was little direct evidence for it. There is however, evidence for a requirement for close spatial association between endoplasmic reticulum and plasma membrane store-operated channels. Jaconi *et al.* (58) utilized centrifugation to redistribute the organellar contents of oocytes and found that entry only occurred in regions with closely apposed endoplasmic reticulum. Patterson *et al.* (59) used drugs to stimulate peripheral actin polymerization, and disrupted communication between Ca^{2+} stores and plasma membrane store-operated channels (but see (60)).

TRP channels

Hardie and Minke (61, 62) first realized that the *Drosophila* photoreceptor calcium channel, TRP, was activated downstream of phospholipase C and might be a candidate for a store-operated channel. When mammalian homologs were cloned ((63) the canonical TRPs, or TRPCs), a number of laboratories pursued this possibility, with initially encouraging results (63-65). However, it soon became apparent

that many of the early findings likely resulted from constitutive activity of over-expressed channels (66, 67). Nonetheless, there are numerous reports of diminished store-operated entry following knock-down of TRPC expression (for example, (68), and others reviewed in (40)). Also, it is clear that under some circumstances, TRPC channels can exhibit store-operated activity when ectopically expressed (69-72).

An important issue is that TRPC channels, when experimentally expressed, do not recapitulate the properties of I_{crac} ; rather, they form non-selective cation channels, or at best channels with only modest Ca^{2+} selectivity. Additionally, they have single channel conductances of conventional size, on the order of tens of picosiemens. Thus, if TRPC channels operated as store-operated channels, it would appear that these channels should have characteristics clearly distinct from I_{crac} (for example, (54), others reviewed in (40)). On the other hand, there is a clear and generally reproducible body evidence showing that TRPC channels can be activated downstream of PLC by mechanisms not involving store depletion, for example, TRPC3, 6 and 7 by diacylglycerols, and there is ample evidence that such a mode of regulation occurs in cells under conditions of physiological regulation (73-77).

The role of Stim1

The role of Stim1 (actually, initially *Drosophila* Stim) in capacitative calcium entry was initially discovered through a limited RNAi screen of thapsigargin-activated Ca^{2+} entry in *Drosophila* S2 cells by Roos *et al.* (78). This was followed shortly thereafter by a report from Liou *et al.* (79) who carried out a limited RNAi screen using thapsigargin-activated mammalian HeLa cells. *Drosophila* has a single Stim gene, while mammalian cells have two, designated Stim1 and 2. In the Roos *et al.* study, following the identification of Stim from the S2 screen, they found that knockdown of Stim1, but not Stim2 reduced store-operated entry and I_{crac} in mammalian cells. However, Liou *et al.* reported a slight reduction in Ca^{2+} entry by knockdown of Stim2 in HeLa cells.

Soboloff *et al.* (80) reported that Stim2 could act as an inhibitor of store-operated entry; however, this action of Stim2 was only seen when expressed at very high levels. Thus the true physiological function of Stim2 may not yet be known.

It seems clear that the function of Stim1 is to act as the initial sensor of Ca^{2+} levels in the endoplasmic reticulum, or the component of it involved in regulation of I_{crac} . The Ca^{2+} sensing domain is an EF-hand that resides in the lumen of the endoplasmic reticulum, N-terminal to the single transmembrane segment. Several laboratories have demonstrated that mutations in the EF-hand region, presumably reducing Ca^{2+} affinity, result in constitutive activation of Ca^{2+} entry with properties expected of I_{crac} (79, 81-83).

Experiments examining the cellular distribution of Stim1 histochemically, or utilizing expression of fluorescent protein fusion constructs have provided intriguing information on the cell biology of this Ca^{2+} sensor. An important finding is that the intracellular distribution of Stim1 does not match well with markers for endoplasmic reticulum. Labeled (YFP) Stim1 appears as fibrillar, or more likely tubular structures in HEK293 cells (Fig. 4) (83), structures not apparent from labeling generic endoplasmic reticulum in the same cell type (84). It is likely that Stim1 will serve to define a distinct organelle, or more likely a distinct compartment within the endoplasmic reticulum. This is gratifyingly consistent with a number of previous studies which concluded

Fig. 4. *Stim1* rearranges following Ca^{2+} store depletion. Confocal images demonstrating that thapsigargin causes rearrangement of YFP-Stim1 from a fibrillar or tubular arrangement into punctae.

that the pool of Ca^{2+} that regulated store-operated channels appears to be a small component of the total endoplasmic reticulum (85-90).

As interesting as the subcellular localization of Stim1 may be, what is more exciting is the idea that Ca^{2+} dissociation causes the protein to redistribute intracellularly. Liou *et al.* (79) first showed that upon Ca^{2+} store depletion Stim1 appeared to redistribute into punctate structures, and move closer to the plasma membrane. This has been confirmed in a number of laboratories (81-83) (Fig. 4). However, a major point of controversy remains regarding the localization of Stim1 when it redistributes into punctae. Liou *et al.* reported that Stim1 moved close to the plasma membrane, but using an antibody directed against the YFP on the N-terminus, they failed to detect Stim1 on the plasma membrane (79). Similar findings were reported by Mercer *et al.* (83) and by Baba *et al.* (91). These latter authors found that N-terminally Flag-tagged Stim1 could not be detected in the plasma membrane of a B-cell line, but still was capable of supporting full store-operated entry. In this case, complete knockout of Stim1 was achieved by targeted gene disruption, leaving no doubt that the N-terminally tagged Stim1 could function in the absence of any residual wild-type protein. And, as will be discussed subsequently, it is possible to greatly increase store-operated entry by co-expression of Stim1 with Orail; however, even these very large I_{crac} -like currents were supported by YFP-Stim1, again with no surface expression detected (83). On the other hand, based on surface biotinylation studies, Zhang *et al.* (81) reported that Stim1 actually translocates into the plasma membrane. A third position was taken by Spassova *et al.* (82) who reported that a fraction of Stim1 was clearly present in the plasma membrane where it appears to function in store-operated entry (based on the inhibition of entry by extracellular application of an anti-Stim1 antibody), but they failed to see any change in the amount of plasma membrane Stim1 following Ca^{2+} store depletion.

The first report on Stim1, prior to its disclosure as an initiator of capacitative calcium entry, identified

it as a surface protein of stromal cells able to interact with B lymphocytes (92), and this surface localization was substantiated in subsequent studies (93, 94). One interpretation of these disparate findings is that native Stim1 is located in the endoplasmic reticulum and in the plasma membrane, but the addition of YFP or a Flag tag to the N-terminus of Stim1 prevents its trafficking to the plasma membrane. This is actually fortuitous, since it allows assessment of the function of Stim1 with a construct restricted to intracellular sites. Thus, one reasonable conclusion is that Stim1 does not translocate to the plasma membrane in response to Ca^{2+} store depletion. And plasma membrane Stim1, although likely present, does not play an obligatory role in activating store-operated channels.

The role of Orails

Orail was originally discovered by Feske *et al.* (95) from a combination of gene mapping of a family with an immunodeficiency attributed to loss of I_{crac} and a whole-genome screen of *Drosophila* S2 cells. Orail is located in the plasma membrane and appears to have four transmembrane domains. However, unlike the case for Stim1, which was included in restricted screens based on its signaling domains, Orail has no recognizable signaling or channel-like domains and thus required the full genome screen. Very soon thereafter, two other groups, also using a similar whole-genome screens of S2 cells, reported on the requirement for Orail (termed CRACM1 by Vig *et al.* (96)) for store-operated entry and for I_{crac} (96, 97). Zhang *et al.* (97) also reported that when *Drosophila* Orail and Stim were co-expressed they appeared to synergize to form unusually large I_{crac} -like currents. This synergy was subsequently confirmed for mammalian Stim1 and Orail by other laboratories (83, 98, 99). I_{crac} current densities were increased up to 50 fold by the combination of Stim1 and Orail, and the resulting currents were indistinguishable from native I_{crac} in both biophysical and pharmacological properties (83, 98, 99). This result indicates that these two proteins can fully recapitulate the properties of I_{crac} , a finding notably missing

in all previous studies of TRP channels; in fact, it is the distinctly different pharmacology or selectivity of TRP channels that revealed that TRP channels could function in a store-operated mode in some instances (69-71, 100). This does not necessarily mean that no other players are involved; however, other proteins functioning in a stoichiometric complex with Stim1 and/or Orai1 would have to be constitutively present in considerable excess.

The large I_{crac} -like currents observed with expression of Stim1 and Orai1 immediately led to speculation that Orai1 was likely the CRAC channel itself, or possibly a subunit of it. However, there are no obvious channel pore-like sequences in Orai1. Three laboratories focused on a string of acidic residues near the extracellular boundary of the first transmembrane domain (101-103). The most interesting mutations targeted a glutamate in position 106 in mammalian Orai1 (or the corresponding residue in *Drosophila* Orai). Mutation to alanine resulted in a non-functional channel; however, the conservative mutation of this glutamate to an aspartate (E106D; E180D in *Drosophila* Stim) resulted in a channel with markedly reduced selectivity for Ca^{2+} . This provides strong evidence that this residue functions as part of the Ca^{2+} binding selectivity filter, and indicates that Orai1 is indeed a pore forming subunit of the CRAC channel. Prakriya *et al.* (101) also investigated a glutamate residue at position 190. Mutation of this glutamate to aspartate or even alanine had no effect on channel function; however, the rather extreme alteration to a positively charged glutamine (E190Q) resulted in diminished Ca^{2+} selectivity. Since subtle effects on channel function were seen with only rather drastic changes in the nature of this amino acid, it may be that this mutation alters the secondary or tertiary structure of the channel, rather than functioning as part of the Ca^{2+} binding site in the channel pore.

In addition to Orai1, mammalian cells have genes for two additional homologs, Orai2 and Orai3. Unlike the case for Stim2, Orai2 appears to function similarly to Orai1, at least when expressed with Stim1 in HEK293 cells (83). Orai2 currents are some-

what smaller, however, and it is not yet known if this is an intrinsic property of Orai2 channels, or indicates a lower level of expression in the overexpression studies. Orai3 currents are even smaller, such that Ca^{2+} currents from co-expression of Stim1 and Orai3 in HEK293 cells are below the limits of detection. However, Orai3-dependent currents can be observed when Na^+ carries the current (unpublished observation), and Orai3 can rescue store-operated entry following knockdown of Orai1 in HEK293 cells (83). Whether Orai2 and 3 form distinct store-operated channels in specific cell types, or function as subunits of heteromeric channels with Orai1 will be a topic of future investigation.

Communication between Stim1 and Orai1

The evidence is very strong that the signaling pathway for capacitative calcium entry begins with the Ca^{2+} sensor, Stim1, and culminates in the activation of channels composed partly or wholly of Orai subunits. The obvious question is how does Stim1 convey information of depleted Ca^{2+} stores to Orai channels? The simplest answer is that when Stim1 coalesces into punctae and approaches the plasma membrane, it directly interacts with Orai channels there. In support of this idea, Yeromin *et al.* (102) reported that *Drosophila* Stim and Orai could be co-immunoprecipitated, and this association was increased by depletion of Ca^{2+} stores. However, Feske *et al.* (95) failed to observe any co-immunoprecipitation of mammalian Stim1 and Orai1. Vig *et al.* (103) reported co-immunoprecipitation of transfected and tagged Stim1 and Orai1, but did not assess effects of store depletion. Nonetheless, it is clear from the work of Luik *et al.* (104) that communication between Stim1 and Orai1 occurs over very short distances; these investigators observed that Orai1 was recruited to sites of Stim1 punctae formation, and that Ca^{2+} entry was spatially restricted to these plasma membrane sites as well. Finally, although a direct interaction between Stim1 and Orai seems the simplest mechanism for signaling from intracellular stores to the plasma membrane channels, there is nothing to rule out the generation of a secondary

message downstream of Stim1 that acts on Orai, for example, the CIF and iPLA₂ pathway described previously (39).

Conclusions

In just the past two years, the discovery of Stim and Orai proteins has revolutionized our thinking about capacitative calcium entry and I_{crac} . It is remarkable how quickly different laboratories confirmed the dependence of I_{crac} on these two genes, and confirmed the ability of these two proteins to recapitulate the long-known properties of CRAC channels. In fact, it could be said that these are the only aspects of the I_{crac} story upon which all (or most) seem to agree. The current ideas about Stim1 and Orai action are consistent with a number of earlier ideas about store-operated entry; for example, the presence of a small, specialized component of the endoplasmic reticulum dedicated to communicating with plasma membrane channels; the observation that biophysical properties of CRAC channels differ substantially from conventional ion channels, predicting that they may also differ substantially in their structure; the general finding that Ca^{2+} refills the stores efficiently without resulting in a rise in global cytoplasmic Ca^{2+} and the suggested close physical association between sites of endoplasmic reticulum signaling and plasma membrane Ca^{2+} entry. One could argue that the original idea of Irvine (57) – conformational coupling – is at least partially vindicated by the current model. However, it appears to be Stim1 rather than IP₃ receptors that are responsible for this coupling. A summary of the store-operated pathway incorporating the functions of Stim1 and Ora1 is shown in Fig. 5. At present there are some general questions that will no doubt be attacked and hopefully resolved in the not too distant future: (i) Are any other players or proteins necessary for activation of capacitative calcium entry and/or I_{crac} ? (ii) What is the role, if any, of plasma membrane Stim1? (iii) What is the composition of native CRAC channels (Orai homo- or heteromultimers; or combinations with TRPs)? (iv) Are there mechanisms of store-operated entry involving other modes of activation

Fig. 5. *Current model for the roles of Stim1 and Ora1.* Agonist activation of a plasma membrane receptor (R) results in formation of IP₃, which activates the IP₃ receptor (IP3R) causing discharge of store Ca^{2+} from a subcompartment of the endoplasmic reticulum. Within this subcompartment, Ca^{2+} binds reversibly to an EF hand motif in Stim1; depletion of Ca^{2+} results in Stim1 without Ca^{2+} bound, which causes Stim1 to redistribute within the endoplasmic reticulum to areas near Orai within the plasma membrane. Stim1 then activates Ca^{2+} -selective Orai channels; the mechanism whereby this activation is accomplished is unknown. Stim1 is also present in the plasma membrane (not shown), although its function there is unclear. TRPC channels can be activated by phospholipase C-derived signals; there is some evidence that they can be store-regulated in a Stim1-dependent manner.

(other than Stim1) and other store-operated channels (other than Ora1)? (v) How do these molecules function in specialized cells and tissues; for example, despite the fact that the capacitative calcium entry story was born of experimental work in salivary glands, the functions of these new molecules and mechanisms in salivary glands have not yet been investigated (or at least, as yet, reported). Nonetheless, the availability of Stim1 and Orai cDNAs will lead to a detailed structural understanding of the various modes of regulation and activation of this pathway. Such information may ultimately prove of use in designing novel pharmacological reagents to aid in the treatment of a number of diseases in which the store-operated entry pathway is thought to play a role.

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