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On the activation mechanism of store-operated calcium channels

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Abstract The development of the patch clamp technique has revolutionised our understanding of the life sciences. One area in which it has made an enormous contribution is cellular signalling. In many cell types, calcium influx across the plasma membrane is essential for the regulation of a wide range of critical physiological responses including secretion, gene transcription and cell growth. For many years the calcium influx pathways in non-excitable cells remained unknown, despite their importance in physiological and pathophysiological states. Very careful and insightful work by James Putney led to the formulation of the capacitative calcium entry (store-operated calcium influx) model, in which the process of emptying intracellular calcium stores resulted in the activation of calcium entry channels. Unequivocal evidence for this revolutionary model was provided by patch clamp studies carried out by Markus Hoth and Reinhold Penner, who demonstrated that store depletion activated a novel class of calcium channel called the CRAC channel. This review provides a historical perspective on the development of store-operated calcium influx and how patch clamping resolved a long-standing controversy in cell physiology. The review also discusses current ideas relating to how store emptying opens channels in the plasma membrane.

Keywords Calcium entry · Calcium channel · Store-operated calcium influx

Introduction

It is a great privilege to be asked to contribute to this historic collection of articles celebrating 25 years of the classic patch clamp paper, published by the laboratories of Erwin Neher and Bert Sakmann in Goettingen 1981. The patch clamp technique has clearly revolutionised our understanding of fundamental processes in the life sciences. One area (of many) in which it has made an enormous impact is the field of cell physiology, particularly in cellular signalling. Although a vast number of hormones, neurotransmitters and paracrine signals impinge on cell-surface receptors, the number of intracellular second messenger pathways that transmit this into cellular responses is remarkably small. Perhaps the most widespread and ubiquitous of all second messengers is Ca^{2+}.

A rise in cytoplasmic Ca^{2+} concentration is used as a trigger for activating a disparate range of responses in virtually all cells throughout the phylogenetic tree [1, 2]. The Ca^{2+} rise stimulates neurotransmitter release, muscle contraction, cell metabolism, cell growth and proliferation as well as cell death through either apoptosis or necrosis. Eukaryotic cells can increase their cytoplasmic Ca^{2+} concentration in one of two ways: release of Ca^{2+} that is compartmentalised within intracellular stores like the endoplasmic reticulum or Ca^{2+} entry into the cell across the plasma membrane [3]. Although Ca^{2+} release, often manifested as repetitive and regenerative intracellular Ca^{2+} oscillations, can activate certain responses, it is Ca^{2+} influx into the cell that is essential for sustaining the activities of most Ca^{2+}-dependent processes.

Ca^{2+} influx is mediated by a range of Ca^{2+}-permeable channels and transporters in the plasma membrane (Fig. 1; [4]). The distribution of the Ca^{2+} channels tends to depend on whether the cell is excitable or non-excitable. In excitable cells, like nerve and muscle, voltage-gated Ca^{2+} channels comprise the preponderate route for Ca^{2+} influx with some contribution from Ca^{2+}-permeable ligand-gated channels [5]. In non-excitable cells on the other hand, voltage-gated Ca^{2+} channels are generally absent. Instead, Ca^{2+} entry is achieved largely by store-operated and, to a lesser extent, second-messenger operated Ca^{2+} channels ([6, 7] Table 1).

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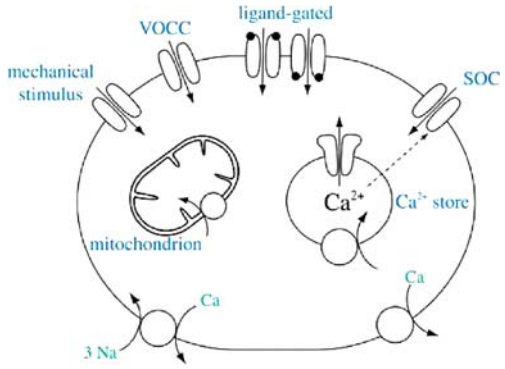

Fig. 1 Plasma membrane-delimited Ca^{2+} transport pathways in eukaryotic cells. Ca^{2+} influx is mediated by a variety of distinct Ca^{2+} channels, which differ in the profile of tissue expression. Ca^{2+} extrusion across the plasma membrane is accomplished through either electrogenic Na^+–Ca^{2+} exchange or the ubiquitous plasma membrane Ca^{2+}ATPase. Under certain conditions, Na^+–Ca^{2+} exchange can reverse, transporting Ca^{2+} into the cell whilst extruding Na^+

Store-operated Ca^{2+} influx

Although the principle of store-operated Ca^{2+} influx was put forward in a seminal review in 1986 [8], its roots lay in a pioneering series of experiments by James Putney in the 1970's and early 1980's. Putney monitored submembranous cytosolic Ca^{2+} through the efflux of $^{86}Rb^+$ and $^{42}K^+$ in parotid acinar cells. He found that muscarinic receptor stimulation evoked a biphasic rise in subplasmalemmal Ca^{2+} concentration: an initial transient phase, reflecting Ca^{2+} release from internal stores, was followed by a sustained rise that was due to Ca^{2+} influx [9]. The Ca^{2+} stores refilled quickly, whereas depleting the stores of Ca^{2+} took considerably longer [10]. A critical finding was that the stores refilled with Ca^{2+} in the absence of an increase in $^{86}Rb^+$ efflux [10]. This led Putney to suggest that receptor-evoked Ca^{2+} influx passed directly into the internal store without first traversing the cytosol. Subsequent work in arterial smooth muscle by Casteels and Droogmans [11] revealed that unidirectional $^{45}Ca^{2+}$ uptake was substantially larger in muscle strips whose stores had been depleted by stimulation with noradrenaline in Ca^{2+}-free solution than in control strips, despite both having been exposed to Ca^{2+}-free solution for the same period of time. This and other works culminated in the formulation of the capacitative calcium entry hypothesis in 1986 by Putney [8]. In this early formulation, the calcium stores were thought to be in direct communication with the extracellular solution. Ca^{2+} release from the stores would automatically cause Ca^{2+} influx into the stores and, if the stores retained a high permeability to Ca^{2+}, then Ca^{2+} entry would pass via the stores into the cytosol. Around this time, two unrelated events became of paramount significance. First, Tsien et al. [12] developed a series of new fluorescent probes (epitomised by fura 2) for monitoring cytosolic Ca^{2+} in living cells in real time. Second, the sequiterpene lactone thapsigargin was identified as a selective inhibitor of the Ca^{2+}ATPase pump on the endoplasmic reticulum [13]. Thapsigargin depleted the stores but without a concomitant rise in $InsP_3$ [13]. When combined with direct recordings of cytosolic Ca^{2+}, it was found that store depletion alone was a sufficient stimulus to initiate Ca^{2+} entry, and agonists activated the same Ca^{2+} influx pathway as thapsigargin and in a non-additive manner [14]. Moreover, store refilling was preceded by a rise in cytosolic Ca^{2+}, indicating that the stores were not in direct communication with the extracellular solution [14]. Collectively, these findings formed the bedrock of store-operated Ca^{2+} entry. The Ca^{2+} content of the stores

Table 1 Biophysical features of the various Ca^{2+} influx pathways reported in non-excitable cells

Current	Conductance	Selectivity	Cell type	References
I_{CRAC}	0.02 pS; 110 Ca^{2+}	$Ba^{2+}>Ca^{2+}>Sr^{2+}$	Mast cell, RBL	[20, 55, 56]
			T cells	[34]
			Hepatocytes	[57]
			Dendritic cells	[58]
			Megakaryocytes	[59]
			MDCK cells	[60]
I_{SOC}	11 pS; 10 Ca^{2+}	$Ca^{2+}>Na^+$	Endothelia	[61]
	1pS; 100 Ca^{2+}	$Ca^{2+}=Ba^{2+}>>K^+$	A431 epidermal cells	[62]
	2pS; 160 Ca^{2+}	$Ba^{2+}>Ca^{2+}$	A431 epidermal cells	[63]
	2.7 pS; 90Ca^{2+}	$Ca^{2+}=Ba^{2+}=Na^+$	Aortic myocytes	[64]
	2,3 pS; 1.5 Ca^{2+}	$Ca^{2+}>Na^+$	Portal vein myocytes	[65]
	5.4 pS; 20 Ca^{2+}	?	Pulmonary artery	[66]
	0.7 pS; 90 Ca^{2+}	?	Mesangial cells	[67]
	43 pS; 1.3 Ca^{2+}	Na^+, $K^+>Ca^{2+}$	Pancreatic acinar cells	[68]
I_{ARC}	?	$Ca^{2+}>Na^+$	HEK293, parotid cells	[69, 70]
$I_{Ca/InsP4}$	2 pS; 100 mM Mn^{2+}	$Ca^{2+}=Ba^{2+}>Na^+$	Endothelia	[71]
I_{NSCC}	5–20 pS; 90 Ca^{2+}	$Ca^{2+}=Na^+=K^+$	Neutrophils	[72]
I_{InsP3}	4–13 pS; 100 Ca^{2+}	$Ca^{2+}\geq Ba^{2+}$	Endothelia, A431	[73, 74]
$I_{GTP\gamma S}$	1–50 pS; 2–100 Ca^{2+}	$Ca^{2+}=Ba^{2+}$	Mast, A431	[19, 75]
$I_{cyclic\ nucleotide}$	?	$Na^+>Ca^{2+}$	Endothelia	[76]

controlled a plasmalemmal Ca^{2+} entry pathway. When the stores were full of Ca^{2+}, plasma membrane permeability to Ca^{2+} was low, but as stores emptied, the Ca^{2+} influx pathway became active.

Ca^{2+} release-activated Ca^{2+} current

Despite its simplicity, store-operated Ca^{2+} influx did not gain widespread acceptance as a general mechanism for Ca^{2+} entry at the time. There were two main reasons for this. First, hardly any single cell electrophysiology had been carried out on non-excitable cells in general. Indeed, it had only been 4 years earlier that Yoshio Maruyama and Ole Petersen [15] had resolved the debate as to whether non-excitable cells indeed expressed ion channels at all, by demonstrating the existence of single channels in pancreatic acinar cells after stimulation with cholecystokinin. Hence, there was still some dispute as to whether non-excitable cells had calcium channels in the plasma membrane. Second, there were other popular models for Ca^{2+} influx in non-excitable cells. Foremost amongst these was the inositol tertrakisphosphate hypothesis, which proposed a central role for $InsP_4$ in evoking Ca^{2+} influx in non-excitable cells [16]. Three years before the formulation of capacitative Ca^{2+} entry, Michael Berridge et al. [17] demonstrated that $InsP_3$ was the long sought after second messenger that released calcium from the endoplasmic reticulum. It was then found that $InsP_3$ could be metabolised to $InsP_4$ by $InsP_3$-3-kinase, leading Robin Irvine [16, 18] to propose an intriguing model in which $InsP_3$ mobilised internal Ca^{2+} and its subsequent conversion to $InsP_4$ elicited Ca^{2+} influx. Although kinetic arguments and the use of thapsigargin tended to provide more support for the capacitative calcium entry model, there was little direct evidence to discriminate clearly between them. A major stumbling block was the difficulty in identifying, electrophysiologically, a Ca^{2+} channel in non-excitable cells.

A major advance came when Reinhold Penner, together with Gary Matthews and Erwin Neher [19] applied the whole cell patch clamp technique combined with fura 2 measurements to study Ca^{2+} entry pathways in mast cells. They found that dialysis with $InsP_3$ resulted in Ca^{2+} influx and both the rate and extent of the cytoplasmic Ca^{2+} signal was determined by the prevalent membrane potential. Hyperpolarisation increased Ca^{2+} entry whereas depolarisation reduced it, as one would expect from consideration of the electrical driving force for Ca^{2+} influx. $InsP_3$-driven Ca^{2+} influx was unaffected by $InsP_4$, but how $InsP_3$ activated Ca^{2+} entry remained unclear. This was resolved in a classic series of experiments by Markus Hoth and Reinhold Penner [20]. They demonstrated that store depletion in mast cells resulted in the activation of a non-voltage-gated, inwardly rectifying, highly selective Ca^{2+} current that they termed Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}). In these experiments, the cytoplasm was dialysed with 10 mM EGTA (or BAPTA), thus ensuring that cytoplasmic Ca^{2+} concentration was clamped at a very low level. Hence, the current could not be Ca^{2+}-activated. Stores were depleted by dialysis with $InsP_3$ or by application of either the Ca^{2+} ionophore ionomycin or the Ca^{2+} ATPase inhibitor thapsigargin. In fact, dialysis with 10 mM EGTA alone was sufficient to empty the stores and activate CRAC channels. The current was remarkably selective for divalent cations like Ca^{2+} [21]. This was a landmark paper. Not only did it demonstrate that store depletion activated a novel Ca^{2+}-selective current, as predicted by the Putney model, but this was the first unequivocal identification of a calcium current in non-excitable cells. Since then, I_{CRAC} has been seen in lymphocytes, rat basophilic leukaemia cells, megakaryocytes, macrophages, MDCK cells and hepatocytes [7]. I_{CRAC}-like currents have been reported in *Xenopus* oocytes, endothelial and epithelial cells [7]. However, biophysically distinct store-operated channels were reported in some other cell types (Table 1), indicating that store-operated Ca^{2+} influx is mediated by a heterogeneous family of ion channels. Nevertheless, I_{CRAC} remains the best characterised and most widely distributed store-operated channel, and much of our understanding of store-operated entry has been derived from studies based on this channel.

In this review, I will focus exclusively on the elusive activation mechanism of store-operated Ca^{2+} channels and describe the various models that have been proposed to explain how store emptying opens these Ca^{2+}-permeable channels.

Activation of store-operated Ca^{2+} influx

CRAC channels are activated by the process of emptying the intracellular Ca^{2+} stores. It does not seem to matter how the stores are emptied since the net effect is the same, namely, opening of CRAC channels [6]. Stores can be depleted by activating cell-surface receptors that couple to phospholipase C or by dialysing cells with $InsP_3$ and related analogues ($InsP_3$-F, Ins2,4,5-P_3 or adenophostin A). Alternatively, stores can be depleted using pharmacological tools like ionomycin or thapsigargin. Finally, simply dialysing cells with high (millimolar) concentrations of Ca^{2+} chelator is often sufficient to empty the stores and activate CRAC channels [6].

How does store depletion result in the activation of I_{CRAC}? In intact cells, store emptying will be accompanied by a rise in cytosolic Ca^{2+} concentration. However, three pieces of evidence argue against a role for elevated cytosolic Ca^{2+} in opening CRAC channels. First, dialysis with Ca^{2+}-containing pipette solutions (200 nM–10 μM) fails to activate I_{CRAC} in the absence of store depletion [22]. Second, high levels of intracellular Ca^{2+} chelator are sufficient to evoke I_{CRAC}. For example, millimolar levels of EGTA or BAPTA clamp cytosolic Ca^{2+} at very low levels (<10 nM), yet I_{CRAC} can develop [20, 23]. Third, the divalent cation chelator TPEN can, in its non-complexed form, cross membranes and access the lumen of the endoplasmic reticulum [24]. Here, it chelates luminal Ca^{2+}, lowering the free Ca^{2+} concentration within the stores but

without evoking Ca^{2+} release. I_{CRAC} subsequently develops [24]. Hence, CRAC channels are not activated by the rise in cytosolic Ca^{2+} that accompanies, at least under physiological conditions, store depletion. Therefore, information about the store Ca^{2+} content has to be conveyed to the CRAC channels indirectly, by an intermediate signalling mechanism. This mechanism requires a Ca^{2+} sensor to detect the fall in intraluminal Ca^{2+} content and then a signal that conveys this information to the channels in the plasma membrane. Despite much research and some interesting leads, this retrograde signalling cascade remains unresolved. Three main models were put forward to account for the activation of store-operated channels: diffusible messenger hypothesis, secretion-like conformational coupling and vesicular fusion (Fig. 2).

Diffusible messenger

The diffusible messenger hypothesis posits a major role for a mobile messenger in linking store content to the channels in the plasma membrane. The messenger may be stored within the endoplasmic reticulum and released into the cytosol after store depletion. Alternatively, it could be generated de novo in the cytosol upon store depletion. Much interest has focussed on a putative messenger released from the stores [25, 26]. Early studies reported the existence of a low-molecular weight factor in an acid-extracted fraction from a Jurkat cell line that evoked Ca^{2+} influx in several different non-excitable cells [25]. The active ingredient was called Ca^{2+} influx factor (CIF). However, subsequent work revealed that CIF-containing extract caused Ca^{2+} release from the stores (which would secondarily trigger Ca^{2+} influx), and its actions were prevented by the muscarinic receptor antagonist atropine [27, 28]. Hence, the acid-extracted fraction seemed to contain a variety of factors capable of generating Ca^{2+} signals. More recent work has attempted to isolate the putative CIF using a series of purification steps [29]. A CIF-containing extract from *Saccharomyces cerevisiae* was shown to activate 3 pS store-operated channels in excised inside-out patches from aortic myocytes [30]. Intriguingly, exposing the patches to permeabilised human platelets whose stores had been depleted with thapsigargin activated the same 3 pS channels [30]. Exposure of the patches to thapsigargin alone, or to permeabilised platelets whose stores were intact failed to activate these channels. Hence, store depletion generates an endogenous factor that diffuses out of human platelets and activates store-operated channels in rat smooth muscle. These same channels were activated by CIF obtained from yeast [30]. If the factor from human platelets is indeed CIF, then this would indicate remarkable conservation in that it is found in yeast and human cells. CIF was proposed to open store-operated Ca^{2+} channels indirectly, via recruitment of Ca^{2+}-independent phospholipase A_2 ($iPLA_2$). Calmodulin binds tightly to $iPLA_2$ at resting levels of cytosolic Ca^{2+}, and this somehow suppresses $iPLA_2$ activity. CIF was found to displace $iPLA_2$ from calmodulin-Sepharose columns, suggesting that CIF activates $iPLA_2$ [31]. Smani et al. [32] also reported that the $iPLA_2$ inhibitor bromoenol lactone (BEL) suppressed activation of store-operated channels in myocytes and I_{CRAC} in RBL cells. $iPLA_2$ hydrolyses membrane phospholipids to generate arachidonic acid and lysophospholipid. Application of lysophospholipid but not arachidonic acid was found to activate the

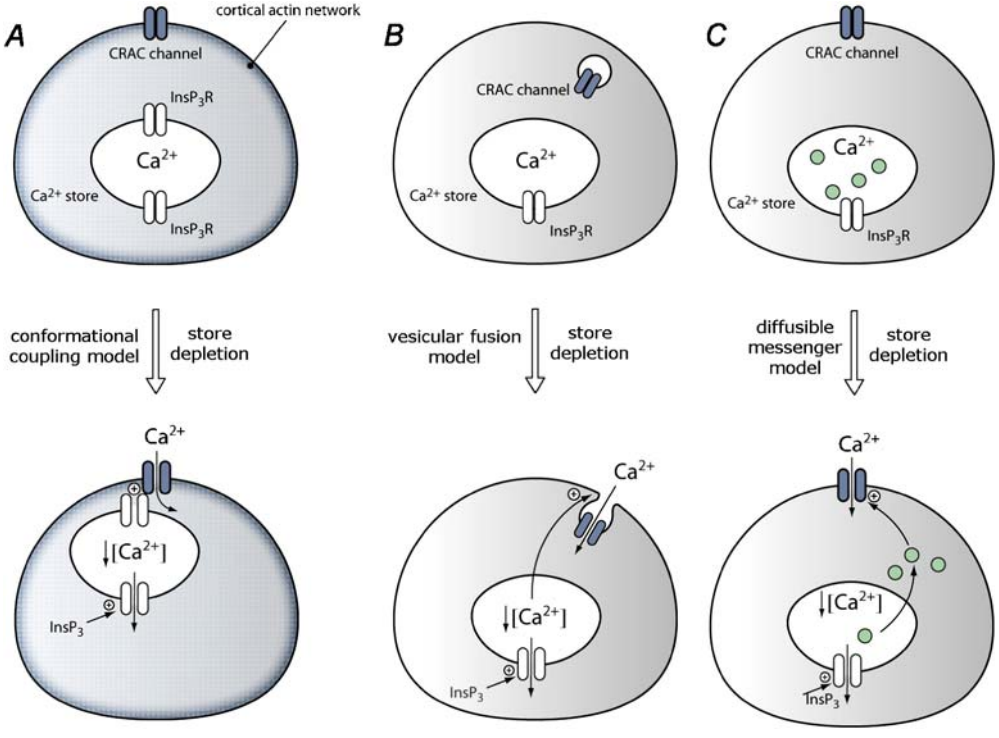

Fig. 2 Cartoon scheme of the various activation mechanisms for store-operated calcium channels. See text for detailed description

3 pS store-operated channels in aortic myocytes [31]. Importantly, lysophospholipid still activated the channels in the presence of BEL, indicating it was downstream of $iPLA_2$ [31].

Nevertheless, there are some concerns with a CIF-type messenger. In some systems, CIF was found to release Ca^{2+} from intracellular stores, raising the concern that its actions may be mediated via store depletion [7]. Moreover, in *Xenopus* oocytes, the CIF-activated Ca^{2+} influx pathway is at least 20-fold less sensitive to La^{3+} than the endogenous store-operated channels [29], suggesting that CIF does not activate the intrinsic store-operated Ca^{2+} influx mechanism. Along these lines, CIF can activate outwardly rectifying non-selective channels in Jurkat T lymphocytes [33] that are not recruited by store depletion with thapsigargin or receptor stimulation [34]. Nevertheless, interesting questions still remain. What is the active ingredient in the extract that activates the channels? How and where is it produced and how is it broken down/removed from the cytosol after store refilling? If CIF displaces calmodulin from target proteins, then it might impact on a variety of calmodulin-regulated processes with quite marked changes in cell function. How is this avoided? Although recent work on CIF is promising, it is important to note diffusible messengers are not limited to CIF. Pharmacological studies have implicated other messengers including NO, 5,6-EET, sphingosine-1-phosphate, small GTP-binding proteins and protein kinases [7]. Nevertheless, until a mobile messenger is formally identified/isolated, the diffusible messenger model remains somewhat speculative.

Vesicular fusion

This model proposes that store-operated channels are not in the plasma membrane at rest but are inserted into the membrane upon store emptying via an exocytotic mechanism. The model does not identify how store depletion promotes exocytosis and, hence, would be compatible with other models described here. The key evidence in support of vesicular fusion was that dominant negative SNAP-25 mutants abolished the store-operated Ca^{2+} current in *Xenopus* oocytes [35], as did botulinum neurotoxin A, which cleaves SNAP-25. Botulinum neurotoxins B and E as well as tetanus toxin were all without effect [35]. In HEK293 cells loaded with fura 2, Ca^{2+} influx after treatment with the SERCA pump inhibitor cyclopiazonic acid was inhibited after injection of botulinum neurotoxin/A1c [36]. In this system, tetanus toxin also inhibited Ca^{2+} influx. However, treatment with brefeldin A for a similar time also impaired Ca^{2+} influx and to a similar extent [36]. Hence, it is not clear whether the effects of the clostridial neurotoxins reflect an action on regulated exocytosis or simply that constitutive vesicular trafficking was impaired. On the other hand, SNAP-25 is not thought to be expressed in non-excitable cells. Rather, it seems confined to neuronal and neuroendocrine systems. Hence, inhibition by botulinum toxin is puzzling because its substrate (SNAP-25) is apparently not present in oocytes or HEK293 cells. Indeed, Scott et al. [37] failed to detect SNAP-25 in either HEK293 or COS-1 cells. However, these cells did express the SNARE protein SNAP-23, which is insensitive to botulinum neurotoxin. Importantly, overexpression of a truncated SNAP-23 mutant failed to affect store-operated influx, although this mutant suppressed cycling of transferrin receptors [37]. Furthermore, expression of a mutant NEM-sensitive factor construct generally inhibited membrane trafficking events, but again failed to interfere with store-operated entry [37]. Along similar lines, dialysis with recombinant truncated α-SNAP protein failed to interfere with either the rate or extent of I_{CRAC} activation in RBL cells even though the same protein inhibited Ca^{2+} -dependent exocytosis [38]. A variety of toxins and pharmacological agents that impair secretory events were all without effect on I_{CRAC}. At least in RBL and HEK293 cells, I_{CRAC} and store-operated Ca^{2+} influx can be dissociated from exocytotic events.

Secretion-like conformational coupling

This model took its roots in the conformational-coupling hypothesis first put forward by Irvine. Irvine postulated that $InsP_3$ receptors on the stores were physically attached to $InsP_4$ receptors in the plasma membrane, and interaction between these two proteins controlled the Ca^{2+} influx pathway with the $InsP_4$ receptor possibly functioning as the Ca^{2+} entry channel [16]. Berridge [39], proposed that $InsP_3$ receptors on the stores were physically coupled to the store-operated Ca^{2+} channels in the plasma membrane. Such a model would be analogous to excitation–contraction coupling in skeletal muscle, where ryanodine type 1 release channels in the sarcoplasmic reticulum are physically coupled to dihydropyridine-sensitive Ca^{2+} channels in the T tubules. The crux of the conformational coupling model is that store depletion alters the conformation of the $InsP_3$ receptor, which then rapidly opens the store-operated Ca^{2+} channels in the plasma membrane [39]. To date, there is no direct evidence in support of this hypothesis. On the other hand, CRAC channels activate slowly after rapid store depletion, developing with a time constant of around 20 s at room temperature [6]. Such kinetics are hard to reconcile with a direct coupling reaction, especially as the analogous system in skeletal muscle activates within milliseconds. To circumvent this kinetic problem, the revised secretion-like coupling model was advanced [40]. In this scheme, store depletion results in migration of the peripheral endoplasmic reticulum to the plasma membrane. When the two membranes are juxtaposed, $InsP_3$ receptors on the stores physically attach to the store-operated channels in the plasma membrane [40]. The movement of the endoplasmic reticulum presumably accounts for the slow kinetics of activation of I_{CRAC}. The coupling reaction is thought to regulated by the peripheral cytoskeleton; stabilisation of this cytoskeleton impedes the coupling from taking place, whereas disaggregation can facilitate binding of $InsP_3$ receptors to the Ca^{2+}

channels. Agents that stabilise the cytoskeleton do interfere with the activation of store-operated Ca^{2+} influx [40, 41]. However, interfering with the cytoskeleton affects numerous ion channels, thereby changing the membrane potential and, hence, electrical driving force for Ca^{2+} entry. In most of the experiments investigating cytoskeletal regulation of store-operated Ca^{2+} entry, the membrane potential was not controlled. In whole cell patch clamp experiments under conditions where the membrane potential was clamped, alterations in the peripheral cytoskeleton failed to affect kinetics or extent of activation I_{CRAC} in RBl-1 cells [42]. Nevertheless, altering the state of the cytoskeleton does interfere with the development of store-operated Ca^{2+} influx in several different cell types, suggesting that it may play a role in regulating store-operated channel activity [43]. Another requirement of the secretion-like coupling model is the need for $InsP_3$ receptors at all stages of store-operated Ca^{2+} influx. Initial experiments with 2-APB, a membrane-permeable $InsP_3$ receptor antagonist, showed that inhibition of $InsP_3$ receptors suppressed store-operated Ca^{2+} influx to thapsigargin even when applied after the Ca^{2+} channels had been activated [44]. However, subsequent studies demonstrated that 2-APB functioned as a CRAC channel blocker, probably acting via an external site on the channels [42, 45, 46]. When $InsP_3$ receptors were inhibited using other antagonists like heparin, I_{CRAC} activated normally [20, 42]. Furthermore, genetic deletion of all three $InsP_3$ receptors in the DT40 B lymphocyte cell line eliminated $InsP_3$ binding as well as $InsP_3$-dependent Ca^{2+} release [46, 47]. However, activation of store-operated Ca^{2+} influx [46–48] and I_{CRAC} were unaffected [45], indicating that $InsP_3$ receptors were not essential to the activation mechanism. However, this does not rule out the possibility that another protein on the endoplasmic reticulum might couple to the CRAC channels. For example, it was suggested that ryanodine receptors might substitute for $InsP_3$ receptors in the DT40 B cell lacking $InsP_3$ receptors [49]. Ruthenium red, an inhibitor of ryanodine-sensitive channels, blocked I_{CRAC} by ~50% in both DT40 wild-type cells and the $InsP_3$ receptor triple knockouts. In RBL-1 cells, on the other hand, I_{CRAC} activated normally in the presence of both heparin and ruthenium red [23].

Enter stromal interaction molecule 1 and 2

A key advancement was provided by the discovery that the protein stromal interaction protein (STIM) is an important component of the activation mechanism and may function as the elusive Ca^{2+} sensor. Cahalan, Stauderman and colleagues exploited the Drosophila S2 cell system to screen for genes involved in store-operated Ca^{2+} influx. They had previously shown that the S2 cells exhibited store-operated Ca^{2+} entry and that the Ca^{2+} influx pathway was very similar in its electrophysiological properties to CRAC channels. Using an RNAi screen directed against 170 genes that had been selected on the basis of channel-like domains, transmembrane regions, Ca^{2+}-binding domains or putative function in store-operated entry, Roos et al. [50] found that knocking down one gene substantially impaired store-operated Ca^{2+} influx and I_{CRAC} in S2 cells. The gene coded for the protein STIM. There are two mammalian homologues of STIM, STIM1 and STIM2, and both seem to be widely expressed. In Jurkat T cells, knockdown of STIM1 substantially inhibited I_{CRAC} development [50]. In HEK293 cells and SH-SY5Y neurobalstoma cells, knockdown of STIM1 also impaired store-operated Ca^{2+} entry. Strikingly, RNAi directed against the closely related STIM2 failed to have any adverse effect on store-operated Ca^{2+} influx [50]. These important findings collectively demonstrated that STIM1 was a conserved component of store-operated Ca^{2+} influx. What is the role of STIM1 in store-operated entry? Overexpression of STIM1 failed to increase the extent of store-operated Ca^{2+} influx, leading Roos et al. [50] to argue that it was unlikely that STIM1 was the channel itself. Indeed, STIM1 is comprised of a single transmembrane-spanning domain, which has no channel-like sequence. However, it is possible that STIM1 is a critical component of a multimeric CRAC channel complex. The protein is found both in the plasma and intracellular membranes, probably the endoplasmic reticulum. Strikingly, the NH_2 terminus, which would be facing the lumen of the endoplasmic reticulum, has an EF-hand domain. Roos et al. [50] pointed out that this could therefore represent the elusive sensor of the ER Ca^{2+} content, which is the initial step in the activation of store-operated Ca^{2+} entry after store depletion. Moreover, STIM1 has motifs (coiled-coli domains and a sterile α motif) that support protein–protein interactions. STIM1 can oligomerise, raising the possibility that the two membranes might couple via STIM1 proteins in the ER and plasma membrane. Expression of STIM1 constructs containing mutations in the EF-hand motif resulted in constitutive Ca^{2+} influx in Jurkat T cells, and this was not associated with any change in store Ca^{2+} content [51]. In resting Jurkat T cells, STIM1 was found to co-localise with SERCA pumps and protein disulphide isomerase, markers of the ER. After store depletion however, STIM1 assumed a more punctuate distribution at the cell-surface, and association with the ER became much weaker [51]. Translocation to the plasma membrane occurred with a time constant of around 5 min, which was only slightly slower than the time course of activation of I_{CRAC}. Quantum-dot labelling of STIM1 revealed a fivefold increase in surface density after store depletion, which was consistent with the increase in STIM1 biotinylation after thapsigargin treatment [51]. Collectively, these results suggest that STIM1 is predominately located in the ER at rest, but lowering luminal Ca^{2+} (via store depletion) or expression of STIM1 EF-hand mutants results in the translocation of STIM1 to the cell surface, where it is subsequently inserted into the plasma membrane. Here, it activates CRAC channels through an unknown mechanism but which could involve direct interaction with the pore-forming subunit, conformational coupling via interaction with ER-based proteins or co-assemble to form functional CRAC channels [51].

A similar siRNA approach was taken by Meyer et al. [52]. These authors screened the database for 2,304 proteins that contained known signalling domains. Using a Ca^{2+} influx assay system, they found that siRNAs targeting STIM1 and STIM2 were able to suppress agonist- and thapsigargin-evoked Ca^{2+} and Mn^{2+} influx in HeLa cells. Overexpression of STIM1 resulted in a significant increase in the extent of store-operated Ca^{2+} entry, and this was blocked by the channel blocker SKF96365 [52]. After overexpression of a YFP-STIM1 construct, Liou et al. [52] found that STIM1 was closely associated with the ER at rest. However, store depletion resulted in a profound redistribution of STIM1 into puncta that were found both inside the cell and near the cell periphery. The puncta could form rapidly, being observable within 60 s. Although some puncta were close to the cell membrane, staining cells expressing YFP-STIM1 with anti-GFP failed to reveal insertion of the YFP-TIM1 protein into the plasma membrane after store emptying [52]. Liou et al. [52] also noted that STIM1 and STIM2 had EF-hand domains likely facing the lumen of the ER. Mutating the first Ca^{2+} binding aspartate residue in the EF-hand to alanine resulted in the formation of puncta even when stores were full, and these were the same puncta as those in which YFP-STIM1 could be found [52]. Expression of the EF-hand mutant resulted in enhanced Ca^{2+} influx even though stores were replete. Total internal reflection fluorescence microscopy revealed that the puncta were located within 100 nm of the plasma membrane, suggesting either a short-range signal involved in the activation of the channels or a coupling-like mechanism involving STIM1 [52].

New results from Gill's laboratory suggest that, in addition to being the ER Ca^{2+} sensor, STIM1 is also in the plasma membrane and modulates the CRAC channel. Spassova et al. [53] expressed a C-terminal deletion mutant of STIM1 lacking protein kinase C and casein kinase II potential phosphorylation sites in Jurkat T cells. They found that rapid inactivation of I_{CRAC}, which occurs in divalent-free solution, was lost. Furthermore, after knock-down of STIM1 in RBL cells, they found that expression of an EF-hand mutant resulted in a change in the pharmacology of the CRAC channels [53]. Instead of a low concentration of 2-APB potentiating I_{CRAC}, it now blocked the current rapidly. Finally and most importantly, an antibody directed against the N-terminal of the EF-hand mutant blocked the development of I_{CRAC} by 70% when applied from the outside [53]. This implies that STIM1 is in the plasma membrane where it is able to regulate certain properties of I_{CRAC}. Could STIM1 have a dual role, in which ER STIM1 and plasma membrane STIM1 perhaps interact to regulate I_{CRAC}?

Although these recent reports identify STIM1 as a critical component of the mechanism activating store-operated Ca^{2+} channels [50–53], there are nevertheless some striking differences. First, in HeLa cells, knocking down either STIM1 or STIM2 reduced store-operated influx, and knocking down both suppressed Ca^{2+} entry [52]. In Jurkat T lymphocytes, knocking down STIM2 had no effect at all [50]. Second, overexpression of STIM1 in HeLa cells resulted in larger store-operated Ca^{2+} entry [52], and when expressed in RBL, cells dramatically increased the size of I_{CRAC} [53]. However, overexpression of STIM1 failed to increase Ca^{2+} influx in HEK293 cells [50].

Third, STIM1 did not seem to be inserted into the plasma membrane upon store depletion in HeLa cells [52], whereas in Jurkat T lymphocytes it was concluded that it was [51]. In RBL cells, the antibody data suggests that some STIM1 may already be in the plasma membrane in the absence of store depletion [53]. An important finding has come from studies on severe combined immunodeficiency patients [54]. T lymphocytes from these patients have dramatically reduced Ca^{2+} influx, an absence of I_{CRAC} and severely compromised T cell activation. Overexpression of STIM1 failed to reconstitute Ca^{2+} influx or I_{CRAC} [54]. Hence, the presence of STIM1 alone is not sufficient for functional store-operated CRAC channels. Very recently, a Drosophila genome-wide search has led two groups to identify the same gene as being central to CRAC channel activation [77, 78]. Knocking down this gene (called Orai1 or CRACM1) in Jurkat T lymphocytes, RBL and HEK293 cells abolished CRAC activity. Importantly, expressing Orai1 in T cells from the immuno-deficient patients reconstituted ICRAC. Orai1/CRACM1 is a plasma membrane protein with four putative transmembrane-spanning domains. Although its exact role in store-operated entry is unclear at present, it could function as the CRAC channel itself, a key regulatory component of a multimeric channel complex or be involved in the activation mechanism, perhaps by interacting with STIM1. Now that the molecular tools are available for addressing these questions, we can look forward to an exciting period as the elusive mechanism of store-operated Ca^{2+} influx is gradually unravelled.

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