REVIEW ARTICLE

Capacitative calcium entry

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INTRODUCTION

The original idea that calcium might enter cells through a capacitative mechanism was first introduced by Jim Putney [1]. The somewhat surprising hypothesis was that calcium entry was regulated by the state of filling of the calcium stores. By analogy with a capacitor in an electrical circuit, the calcium stores prevent entry when they are charged up but immediately begin to promote entry as soon as stored calcium is discharged. This capacitative entry mechanism is present in many cells and has properties which are very similar from one cell to the next [2–6]. Capacitative calcium entry can be switched on by a great variety of stimuli such as normal agonists or pharmacological agents all of which share a common property of releasing stored calcium. Examples include calcium-mobilizing agonists [7–13], the calcium-mobilizing second messenger inositol 1,4,5-trisphosphate (InsP_3) [11,12,14–18], the calcium ionophore ionomycin [14,19], inhibitors of the endoplasmic reticulum (ER) pumps such as thapsigargin [8,10,12,20] and cyclopiazonic acid [20,21] or simply by incubating cells in Ca^{2+}-free conditions [12,14,15]. All this evidence indicates that the entry of external calcium is somehow controlled by the calcium content of the ER. This review is focused primarily on the mechanism of capacitative Ca^{2+} entry.

CHARACTERISTICS OF CAPACITATIVE CALCIUM ENTRY

Functional significance

There is a growing awareness that this capacitative calcium entry plays a central role in many aspects of cell signalling. For example, the Ca^{2+} oscillations which have been recorded in many cells are maintained by the small but constant influx of external calcium [22,23]. Capacitative calcium has also been implicated in the function of osteoclasts [24], the regulation of adenylate cyclase [25], regulatory volume decrease [26], phototransduction in *Drosophila* photoreceptors [27–32] and the activation of mitogenesis in lymphocytes [10] and in fibroblasts [33]. Control of proliferation is a particularly interesting example because certain forms of immunodeficiency may result from a defect in calcium entry [34]. Also, there was a marked increase in capacitative calcium entry in simian virus 40 (SV-40)-transformed Swiss 3T3 cells [35]. With such an important role in signalling it is not surprising that there is such interest in trying to unravel the mechanism of capacitative calcium entry.

The calcium-release-activated calcium (CRAC) channel

To distinguish it from other calcium entry channels, Hoth and Penner [14] coined the term calcium-release-activated calcium current (I_{CRAC}) to refer to the current flowing through these capacitative calcium entry channels. The permeation pathway for I_{CRAC} is unusual in having a very low unitary chord conductance with estimates as low as 24 fS [10]. The entry pathway is estimated to conduct about 11000 Ca^{2+} ions/s. By comparison, the InsP_3 receptor would have conducted approx. 1.5×10^6 Ca^{2+} ions in the same interval of time [36]. Although the very low permeation rate is compatible with there being a carrier, noise analysis seems to indicate that I_{CRAC} passes through a channel [10]. It is important to stress, however, that the nature of the permeation mechanism is still unknown. For convenience, it will be referred to as a CRAC channel, mindful of the fact that this remains to be proven. The channel is blocked by trivalent and bivalent ions with an efficacy sequence of $La^{3+} > Zn^{2+} > Cd^{2+} > Be^{2+} = Co^{2+} = Mn^{2+} > Ni^{2+} > Sr^{2+} > Ba^{2+}$ [15].

Perhaps the single most important property of capacitative Ca^{2+} entry is its sensitivity to Ca^{2+} [11,12,14,15,27,28,37–39]. Calcium seems to have a biphasic effect in that it can both activate and inhibit the influx pathway. Most of the evidence concerns the calcium-dependent inactivation of entry, but studies on *Drosophila* photoreceptors suggest that calcium may also exert a positive effect during the activation process [27]. In order to overcome the calcium-dependent inactivation, I_{CRAC} is best recorded when the inactivation process is prevented by perfusing the cell with calcium chelators [14,15,39]. The faster chelator 1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) was much more effective than EGTA in maintaining the current, indicating that the inactivation site is located very close to the mouth of the CRAC channel [39]. This sensitivity to Ca^{2+} is responsible for the complex kinetics of Ca^{2+} entry which will feature significantly in the subsequent discussion of the capacitative entry models.

Identification of the CRAC channel—is it *trp*?

As yet, the CRAC channel has not been identified. However, it has been suggested that the transient receptor potential (*trp*) gene product in *Drosophila* photoreceptors might function as such a capacitative calcium entry channel [14,15,29,32]. In keeping with its proposed role in calcium entry, the *trp* gene was found to display significant amino acid sequence similarity to voltage-dependent calcium channels [40]. The *trp* phenotype is characterized by an inability of the photoreceptors to sustain an influx of Ca^{2+} during intense illumination [41]. However, these *trp* mutants do produce a phasic response which is probably mediated by a separate channel such as the *trp-like* (*trpl*) channel [40]. When transfected into Sf9 cells, the *trp* channel was sensitive to store depletion [42] but *trpl* was not [43]. However, the latter was sensitive to receptor stimulation and may be activated either by G-proteins or by second messengers such as InsP_3 inde-

Abbreviations used: BAPTA, 1,2-bis-(o-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid; CIF, calcium influx factor; CRAC, calcium-release-activated calcium; DHPR, dihydropyridine receptor; ER, endoplasmic reticulum; GTP[S], guanosine 5-'[γ-thio]triphosphate; I_{CRAC}, CRAC current; LIC, light-induced inward Ca^{2+} current; PKC, protein kinase C; PLC, phospholipase C; RYR, ryanodine receptor; SMOC, spontaneous miniature outward current; SR, sarcoplasmic reticulum; *trp*, transient receptor potential; *trpl*, *trp-like*; norpA, no receptor potential.

pendently of store depletion [43,44]. Homologues of *trp* have now been described in man [44a], mouse [44b] and *Xenopus* [44b], thus providing further evidence that *trp* might be the functional analogue of the mammalian CRAC channel. However, these two channels have very different permeability properties. The *trp* channel has a higher conductance than the CRAC channel and is also much less specific. Nevertheless, there are sufficient functional similarities between the CRAC channel and *trp* to indicate that a study of *Drosophila* photoreceptors may help to throw some light on the mechanism of capacitative calcium entry [31].

CAPACITATIVE CALCIUM ENTRY MODELS

The problem is to explain how information is transmitted across the gap separating the ER from the plasma membrane. The various models that have been put forward can be divided into those that propose the existence of a diffusible factor and those that consider information is transferred more directly through a protein–protein interaction [5,6,45].

Models based on diffusible factors

G-protein models

Capacitative Ca^{2+} entry can be modulated by injecting cells with either guanosine 5′-[γ-thio]triphosphate (GTP[S]) or guanosine 5′-[β-thio]bisphosphate (GDP[S]) [46–49]. The interpretation of such experiments is difficult because such non-metabolizable guanine nucleotides are likely to interfere with a number of G-protein-linked events and this may account for some of the contradictory effects that have been described following injection of GTP[S]. There are clear indications that some of the stimulatory effects of GTP[S] can be ascribed to activation of the heterotrimeric G-proteins responsible for activating phospholipase C (PLC) to generate InsP_3, leading to store depletion and the onset of entry [46–49]. Such a notion is supported by the fact that these effects can be duplicated by treating cells with AlF_4^- [47–49]. Such stimulation can be followed by inhibition which may result from the parallel formation of diacylglycerol activating protein kinase C (PKC) [49]. There are indications that capacitative calcium entry is inhibited by PKC [12,50,51].

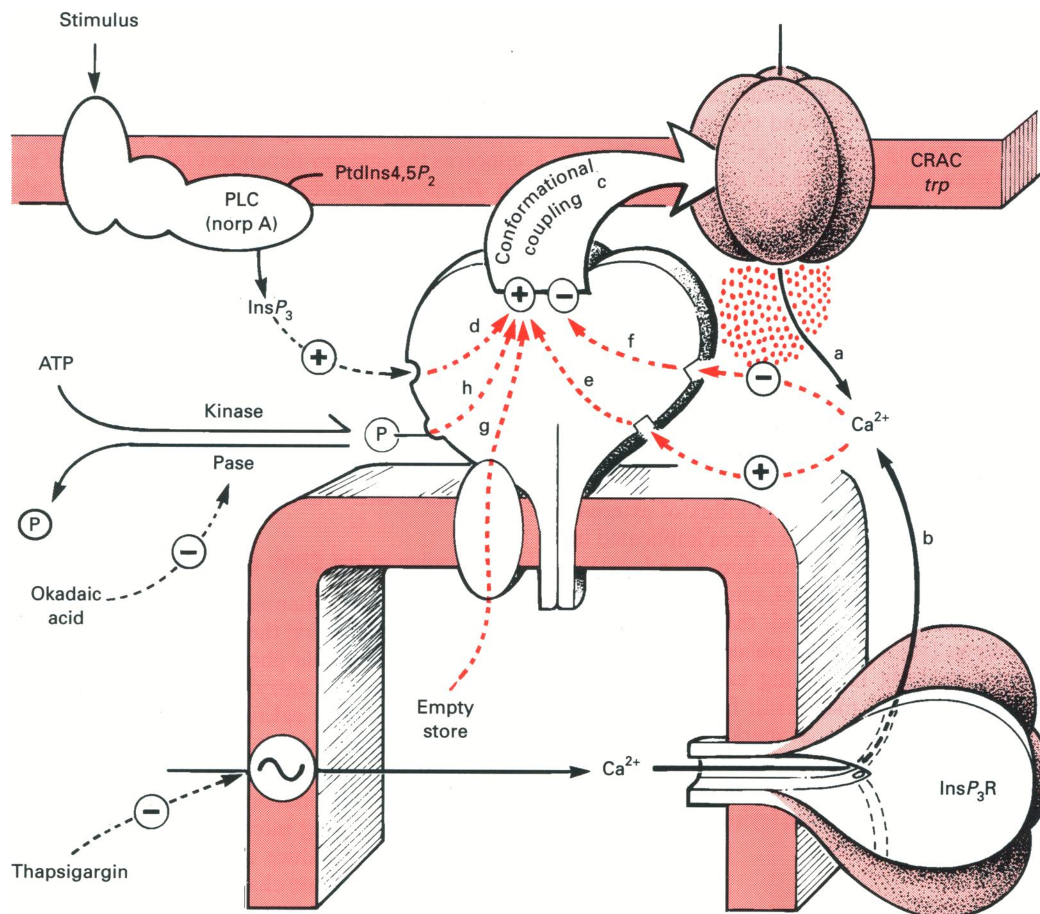

Figure 1 Conformational coupling model for capacitative calcium entry

The different components are drawn roughly to scale to illustrate the problem of transferring information across the 20 nm gap separating the ER from the plasma membrane. The specific proposal is that the large head structure of the InsP_3 receptor (InsP_3R) integrates a variety of both positive and negative signals that are then transmitted to the CRAC channel through a conformational coupling mechanism. Pathways: a, Small Ca^{2+} current through the CRAC channel. It creates a microdomain of Ca^{2+} (stippling) capable of activating binding-sites up to 4 nm away. b, Large Ca^{2+} current through a neighbouring free InsP_3 receptor. c, Information transfer through conformational coupling. d, One of the primary regulators of conformational coupling is the InsP_3 produced by calcium-mobilizing stimuli. e, Low concentrations of Ca^{2+} operate a positive feedback loop capable of enhancing the conformation that favours entry. f, High concentrations of Ca^{2+} operate a negative feedback loop that blocks Ca^{2+} entry. g, Emptying the store can induce the active conformation of the InsP_3 receptor independently of InsP_3. h, Phosphorylation of the receptor can modulate the changes in conformation.

However, there is also evidence that the inhibitory effect of GTP[S] might depend upon the disruption of a small G-protein which may play a more direct role at the level of calcium entry [45–47]. Some of the proposed functions include a role as a diffusible messenger [47], part of an ER mechanism for generating a diffusible messenger [46,47], part of a mechanism for responding to a diffusible messenger [46], or mediating a vesicle fusion event which may insert CRAC channels into the plasma membrane [47]. A possible role in conformational coupling will be described later.

Calcium influx factor (CIF)

Another model considers that the ER, when emptied of its calcium, begins to release a factor which then diffuses to the membrane to open I_{CRAC} [9,52,53]. This factor has been named CIF and is postulated to be a low-molecular-mass phosphorylated compound that is stored in the ER awaiting the onset of store depletion for its release [52]. There also are indications that protein phosphatases may regulate the responsiveness of the entry channel to CIF, implying that the latter may act by promoting channel phosphorylation. Phosphatase inhibitors such as okadaic acid, cyclosporin and calyculin A were able to enhance the responsiveness of cells to threshold levels of either CIF or the calcium-mobilizing agents thapsigargin and carbachol [54]. Inactivation of entry by Ca^{2+} is explained on the basis of a Ca^{2+}-dependent activation of the enzymic cascade that degrades CIF.

The first evidence for CIF was based on measuring Ca^{2+} signals after adding crude cellular extracts to intact cells [52,53]. Although these results have been reproduced, they were given an alternative interpretation [55] which was not accepted by the original authors [56]. When tested on certain cells (e.g. lacrimal cells and hepatocytes) the extract containing the putative CIF mobilized internal calcium through a mechanism that was blocked by both heparin and by atropine, suggesting that it was acting on an external receptor to generate InsP_3 [57]. A similar conclusion was reached from studies on *Xenopus* oocytes where external application of the extract produced a response only in those oocytes which were also sensitive to methacholine [57]. Some of the controversy might be resolved by the observation that the crude extract seems to contain at least two factors, one which can act from the outside and the other which only works when injected [58]. The latter has properties similar to that described originally, i.e. it is a small molecule (< 1000 Da) whose activity is abolished by alkaline phosphatase treatment, which suggests that it is a small phosphorylated compound [58]. One of the interesting properties of this intracellular CIF is that its action is markedly potentiated by okadaic acid, indicating that it may act through a serine/threonine kinase pathway [58]. Clearly the identification of CIF is an urgent prerequisite in order for this model to progress further.

Models based on protein–protein interactions

Information transfer through protein phosphorylation and dephosphorylation

It has been proposed that the ER might possess protein kinases or phosphatases capable of altering the phosphorylation state of the entry channels [15]. However, much of the information is contradictory. For example, there are a number of reports indicating a role for protein phosphorylation in capacitative calcium entry [15]. As described in the previous section, phosphatase inhibitors which enhance serine-threonine phosphorylation will enhance entry [9,58]. On the other hand, activation of PKC exerts an inhibitory effect [12,50,51]. A possible explanation for these inconsistencies is that the separate kinases phosphorylate different components of the entry mechanism. In Figure 1 the kinase is shown to be phosphorylating the InsP_3 receptor, but the CRAC channel is also a likely target for protein phosphorylation.

Perhaps the most consistent information concerns tyrosine phosphorylation, which has been implicated in coupling store depletion to calcium entry [59–63]. The tyrosine kinase inhibitor genistein was found to inhibit entry into fibroblasts [33], rat pancreatic acinar cells [64], lymphocytes [65], blood platelets [60–62] and endothelial cells [66]. Since some of these cells use tyrosine kinase-linked receptors to activate PLC, some of the actions of genistein may depend upon a reduction in the level of InsP_3. However, genistein was capable of inhibiting entry induced by thapsigargin, suggesting a more direct role for tyrosine phosphorylation in calcium entry [33,60,61,66]. The specific proposal is that store depletion regulates a tyrosine phosphatase embedded in the ER [59,60–63]. What is not clear at this stage is how the enzymes embedded in the ER transmit information across the gap between the membranes. One suggestion is that information may be transmitted by means of a 130 kDa protein [59]. Consistent with the conformational coupling model described in the next section, the InsP_3 receptor may also be a candidate for transmitting information because it becomes tyrosine phosphorylated during the activation of T-cells [67].

Conformational coupling

The conformational coupling model proposes that information transfer is mediated by the InsP_3 receptor functioning as the go-between of the two membrane systems [22,68]. One version of the original model emphasized the possible role of InsP_4 in the coupling processes [68], whereas the other placed more emphasis on the role of store emptying [22]. Since much recent experimental evidence has been interpreted on the basis of this model [12,29–32], it would be appropriate to reappraise it and to extend it to incorporate some of the latest information. The specific proposal is that the signals that regulate capacitative Ca^{2+} entry are integrated by the large cytoplasmic head of the InsP_3 receptor which then transmits the information directly to the CRAC channel through a protein–protein interaction (Figure 1). As illustrated in Figure 1, it is envisaged that InsP_3 receptors can have two quite distinct functions; they can either release Ca^{2+} to the cytosol (Figure 1, pathway b) or they can use their large cytoplasmic heads to transmit information to the surface channel (Figure 1, pathway c). Given that there are separate isoforms of the InsP_3 receptor, it is possible that these two signalling functions might be performed by different receptors. Stable transfection of Jurkat cells with antisense type-I InsP_3 receptor cDNA resulted in cells which failed to generate the normal receptor-mediated calcium signal based on calcium mobilization from the internal stores [69]. The fact that normal calcium entry was observed when the stores were discharged artificially with thapsigargin seems to rule out a role for the type-I InsP_3 receptor in conformational coupling [69]. However, the calcium phenotype of these Jurkat cells might be explained if the entry is controlled by either the type-II or type-III InsP_3 receptors. Since the antisense probe probably also eliminated the type-II InsP_3 receptor [69], it seems likely that conformational coupling might be mediated by the type-III receptor. Some support for such a role has come from studies on *Xenopus* oocytes where over-expressing the type-III receptor had no effect on InsP_3-induced calcium release but it markedly enhanced calcium entry (S. DeLisle, personal communication).

It seems therefore that the process of calcium release (Figure 1, pathway b) might be mediated by the type-I receptor, whereas entry is mediated by a separate receptor, perhaps type III. Also it is necessary to consider that free InsP_3 receptors (type I) located near the plasma membrane may be able to gate sufficient Ca^{2+} (Figure 1, pathway b) to influence the operation of the coupling complex.

The exact nature of the protein–protein coupling between the InsP_3 receptor and the CRAC channel is unknown. In the case of the *trp* protein, a unique proline-rich region in the hydrophilic C-terminal tail could play some role in the proposed interaction with the InsP_3 receptor [42]. Evidence implicating G-proteins in Ca^{2+} entry (as described earlier) could be accommodated in this model by proposing that they may facilitate the conformational transfer of information from the InsP_3 receptor to the CRAC channel (Figure 1). In this respect, it is interesting to note that a recently identified InsP_4-binding protein appears to be a member of the family of GTPase-activating proteins perhaps related to the activation of *ras* [70]. It is conceivable, therefore, that the proposed role of InsP_4 in Ca^{2+} entry [68] might be explained through an action on a putative G-protein involvement in conformational coupling.

The main components of the conformational coupling mechanism are the structural elements (the InsP_3 receptor and the CRAC channel) and the control elements (Figure 1, pathways d to h) that act on the InsP_3 receptor to induce the change in conformation (Figure 1, pathway c). In the absence of other candidates, it is proposed that the head of the InsP_3 receptor is responsible for transferring information between the two membranes. However, it is also possible that other proteins might play such a role, as has been proposed for the junctional foot proteins (triadins) in conformational coupling in skeletal muscle [71]. A key feature of the conformational coupling model is that the InsP_3 receptor 'senses' when the store is empty and can then transmit this information to the CRAC channel (Figure 1, pathway g). There already is considerable evidence that the calcium-releasing activity of the InsP_3 receptor can be influenced by the calcium content of the lumen [72]. Since those parts of the InsP_3 receptor that face the lumen appear to lack obvious calcium-binding sites, the sensing of luminal calcium must be carried out by other proteins such as calsequestrin or calreticulin. Indeed there already is evidence for reciprocal interactions between calsequestrin and the ryanodine receptor (RYR) [73,74]. It is proposed, therefore, that as calcium drains out of the ER, proteins such as calsequestrin or calreticulin undergo a conformational change which is then transferred first to the InsP_3 receptor and then to the CRAC channel.

An important structural feature of this model is that the large cytoplasmic head of the InsP_3 receptor is closely apposed to the CRAC channel in the plasma membrane such that information can be transferred through a direct protein–protein interaction. When this model was first introduced [68], the analogy was drawn with the process of excitation-contraction in skeletal muscle where the dihydropyridine receptor (DHPR) is thought to act directly on the RYR [75]. The normal Ca^{2+} conductance of DHPR is suppressed when it is coupled to the RYR. Such an arrangement makes sense because Ca^{2+} entry through the latter would rapidly desensitize the RYRs thereby curbing Ca^{2+} release from the sarcoplasmic reticulum (SR). It is interesting to speculate that the Ca^{2+} release function of the InsP_3 receptor might also be switched off when it is coupled to the CRAC channels. As described in the next section, this inhibition of the InsP_3 receptor may result from its binding to ankyrin. Since its conductance is so much larger than the CRAC channel (as described earlier), the large Ca^{2+} current flowing through the InsP_3 receptor would dominate the ensemble by creating a local high concentration of Ca^{2+} to inhibit entry. Therefore, it is proposed that the InsP_3 receptor may be uncoupled from its release function when it is acting to control entry. Some evidence for this has come from work on *Drosophila* photoreceptors where InsP_3 can activate Ca^{2+} entry without causing detectable release [76,77]. The release of Ca^{2+} may have been missed because the stores in these photoreceptors are very small and confined to a narrow band immediately below the rhabdomere. It should be stressed, however, that suppression of the Ca^{2+} release function of the InsP_3 receptors is not necessary for the operation of the conformational coupling mechanism. Indeed, studies on the *Drosophila* photoreceptors mentioned earlier suggest that the InsP_3 receptor might be able to activate entry through two separate mechanisms. For example, the InsP_3 receptor might be capable of coupling either to a channel such as *trpl* without requiring store depletion (i.e. pathway d in Figure 1), or to a channel such as *trp* which will depend on prior depletion (i.e. pathway g in Figure 1) [31,40]. While the former is not strictly capacitative calcium entry it could represent an important mechanism of calcium entry.

An important refinement of the earlier conformational coupling models concerns the attention now focused on the possibility that the InsP_3 receptor might function as an integrator of the positive and negative control elements that regulate capacitative calcium entry [27,31] (Figure 1). Another new idea is that emptying the store may not be the only mechanism for switching on Ca^{2+} entry through such a conformational coupling mode. Other factors include InsP_3, Ca^{2+} (positive and negative) and protein phosphorylation. What the model in Figure 1 tries to stress, therefore, is that control of entry might be multifactorial.

ER/plasma membrane association

For the conformational coupling model to work, the ER must lie sufficiently close to the plasma membrane for the InsP_3 receptor and the CRAC channel to interact with each other (Figure 1). Evidence for such an association has come from cell fractionation studies which revealed that InsP_3 receptors often appear in the plasma membrane fraction [78–81]. Further fractionation revealed that these InsP_3 receptors were located in an ER membrane component that was tightly bound to the plasma membrane but could be dissociated by treatments which disrupt the cytoskeleton [79,81]. It has been proposed, therefore, that the cytoskeleton might provide a scaffold to hold the ER near the plasma membrane [79,81]. Binding sites for ankyrin, which functions as an adaptor protein connecting integral membrane proteins to the cytoskeleton, have been detected on the InsP_3 receptor in brain [82] and in T-lymphoma cells [83,84], suggesting that the InsP_3 receptor could function to bond the two membranes together. Indeed, Joseph and Samanta [82] have proposed that this link to the cytoskeleton could play a role in capacitative calcium entry in line with the conformational coupling model depicted in Figure 1. Alternatively the cytoskeleton could play a more direct role in transmitting the conformational change in the InsP_3 receptor to the CRAC channel in the plasma membrane [6]. Relevant to this idea is the finding that the *trp* protein also has an ankyrin-binding motif [31,40], which could provide a cytoskeletal link to the InsP_3 receptor.

An interesting feature of ankyrin binding to the InsP_3 receptor is that it inhibits InsP_3-induced release of calcium [83,84]. This inhibitory effect of ankyrin is consistent with the proposal outlined earlier that the calcium-mobilizing role of the InsP_3 receptor might be inactivated when it functions in a con-

formational coupling mode. The ankyrin-binding domain of the InsP_3 receptor has been located near the C-terminus which sticks out into the cytoplasm [84]. This C-terminal region of the InsP_3 receptor plays a critical role in calcium release because it represents the site of action of both inhibitory and stimulatory antibodies [23].

Since the InsP_3 receptor is a large molecule with a diameter of 10–13 nm [85] (as observed in electron micrographs of purified negatively stained receptors), it should be apparent in conventional electron micrographs. For example, the molecular complexes between the ryanodine and dihydropyridine receptors in skeletal muscle described earlier have been visualized as foot structures that span the gap between the T-tubule and SR membranes [75,86]. These feet have a diameter of about 25 nm and are tightly packed together (centre-to-centre distance of approx. 30 nm) to form characteristic striations in the junctional region of the triad. The head of the RYR plays an important role in determining the size of the gap because in mutant mice, where the RYR is absent, the gap is reduced by almost a half [87]. Given that the InsP_3 receptor is somewhat smaller than the RYR, one might expect the gap determined by the coupling complex to be smaller. This coupling complex involving the InsP_3 receptor (Figure 1) will be more difficult to visualize, mainly because they are likely to be present at much lower densities. A current estimate is that the CRAC channels in lymphocytes may be separated from each other by about 140 nm [39]. Nevertheless, foot structures resembling those in muscle have been described in *Xenopus* oocytes where they span the 8–13 nm gap between the plasma membrane and closely apposed sacs of ER [88]. These densities have an average width of 17 nm (S.D. = 5 nm) which is very close to the diameter of the InsP_3 receptor (10–13 nm) [85]. As the dimensions of these bridging structures are consistent with the molecular complex depicted in Figure 1, they could be responsible for the capacitative Ca^{2+} entry that has been recorded in these oocytes [7–9,12,17]. In blowfly photoreceptors, the ER also comes into very close contact (9 nm) with the bases of the microvilli of the rhabdomere [89]. In some of the images (see Figure 2 in reference [89]) there are clear indications of periodic densities linking the two membranes with dimensions very similar to those described in *Xenopus*. Consistent with the proposed coupling between the InsP_3 receptor and *trp*, the latter has been localized, using immunohistochemical staining, to the base of the microvilli in close apposition to the InsP_3-sensitive calcium stores [90].

Further support for the idea that capacitative Ca^{2+} entry depends upon the plasma membrane being closely juxtaposed to the ER has come from studying Ca^{2+} influx in mitotic cells [91,92]. Interphase HeLa cells respond to histamine with the typical sequence of Ca^{2+} release followed by Ca^{2+} entry. During metaphase, however, the persistent entry phase is absent, indicating loss of the normal coupling between depletion of the InsP_3-sensitive Ca^{2+} store and the subsequent influx of external calcium. This uncoupling might be explained by the fact that the reticular ER system which normally extends throughout the cytoplasm during interphase is withdrawn into a tightly packed mass concentrated around the spindle during mitosis [93]. If entry is controlled by conformational coupling (Figure 1), such a retraction of the ER would pull the InsP_3 receptors away from the plasma membrane Ca^{2+} channels thereby disrupting the entry mechanism.

Mechanisms of graded calcium entry

Since calcium entry can be graded [94], it is necessary to consider how the entry signal can vary with stimulus intensity. This graded entry can easily be accommodated by the diffusion models by proposing that the production of diffusible messengers such as CIF vary depending on the level of store depletion. It is less easy to imagine how the conformational coupling mechanism might be graded. One possibility is that entry might be 'quantal' as has been described for the internal release of calcium from single cells [95]. As stimulus intensity increases, stores may begin to release their calcium in an all-or-none manner, but in a sequential manner depending on their variable sensitivities. The rate of entry will then be graded depending on the proportion of stores that have been emptied and have thus switched on their associated capacitative calcium entry mechanism. One way in which this system might be organized is for the generation of InsP_3 to be punctate, i.e. at limiting agonist concentrations very few receptors will be activated resulting in the elevation of InsP_3 and the activation of entry within small microdomains. As the concentration increases, the number of these microdomains will increase resulting in a smoothly graded activation of entry. As described later, the bumps in *Drosophila* photoreceptors are examples of such unitary entry events. Future studies will have to pay particular attention to how the entry of calcium into cells is graded.

Feedback regulation through calcium

The most important new element introduced into the conformational coupling model is the role of Ca^{2+} both as a positive (Figure 1, pathway e) and a negative regulator (Figure 1, pathway f) of entry. Recent studies on Jurkat T-lymphocytes have revealed that the negative feedback effect of Ca^{2+} is highly localized, in that it operates at a site located 3–4 nm from the mouth of the CRAC channel [39]. Zweifach and Lewis [39] concluded, therefore, that the Ca^{2+}-binding site must be on the Ca^{2+} channel itself. Another possibility, as postulated in the model in Figure 1, is that the InsP_3 receptor may also contribute to Ca^{2+} sensitivity [27,31] since it is known to contain sites for both positive (Figure 1, pathway e) and negative (Figure 1, pathway f) feedback. Such calcium-sensitive sites on the InsP_3 receptor could function either as alternative or as additional sites of regulation to those thought to be on the entry channel itself. In its conformational coupling mode, the InsP_3 receptor will certainly come within the proposed 3–4 nm Ca^{2+} microdomain emanating from the mouth of the CRAC channel (see the stippling on Figure 1). Furthermore, the biphasic effect of Ca^{2+} on the InsP_3 receptor [96–101] exactly matches the positive and negative feedback effects of Ca^{2+} on the capacitative entry mechanism. A similar biphasic effect of calcium has been described for the conformational coupling mechanisms in skeletal muscle that depends upon the interaction between the DHPR and the RYR [102]. At low concentrations calcium promotes release but becomes inhibitory at higher concentrations.

The relationship between the rate of Ca^{2+} entry and the ambient Ca^{2+} concentration will be represented by a bell-shaped curve which has two minima and a single point poised between the activation and inactivation phases where entry will be maximal (Figure 2). Note that as the Ca^{2+} rises, the rate of Ca^{2+} entry will pass through an activation followed by an inactivation phase. Depending on how quickly the inactivation phase reverses, the entry process will track back through a reciprocal activation/inactivation sequence as the Ca^{2+} concentration returns to the initial minimum value (Figure 2). The existence of the positive and negative loops suggests that entry is modulated through an autoregulatory system which ensures that once entry has been switched on, either by InsP_3 or by store depletion, the conformational coupling mechanism will attempt to drive itself

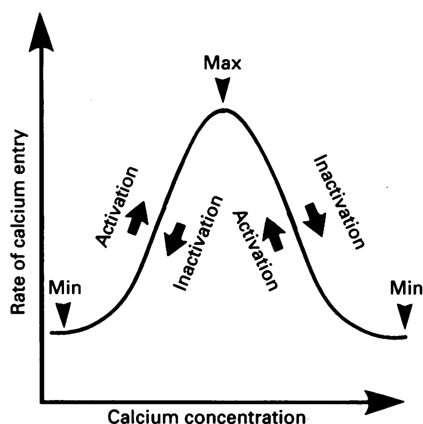

Figure 2 Theoretical biphasic activation curve for capacitative Ca^{2+} entry

See text for further details.

towards an equilibrium position where the rate of entry through the CRAC channel will be as close as possible to its operational maximum. In order to understand how this molecular complex operates, therefore, it is essential to consider the Ca^{2+} concentration within the microdomain surrounding the entry complex [39]. It is conceivable that the conductance of the CRAC channel is such that it delivers Ca^{2+} at just the right rate such that the level of Ca^{2+} within the microdomain will optimize the activity of the transducing complex. Much of what is known about capacitative Ca^{2+} entry, particularly its kinetic properties, can be explained by this conformational coupling model.

KINETICS OF CAPACITATIVE CALCIUM ENTRY

Capacitative calcium entry has very distinctive kinetic properties which provide valuable information on the mechanism of entry and may help to decide between the different models described earlier. Whatever mechanism functions to mediate entry, it will have to accommodate these characteristic kinetic features of capacitative calcium entry.

Latency

Some of the earlier studies using the Mn^{2+} quench technique to monitor Ca^{2+} entry revealed that the influx component was often delayed relative to the mobilization transient [21,103,104]. In the case of the avian salt gland, the onset of entry occurred 20–30 s after the peak of the release phase [104]. Such long delays in the activation of entry have also been described in pancreatic acinar cells [105] and *Xenopus* oocytes following stimulation by calcium-mobilizing agonists such as serotonin [7], lysophosphatidic acid [12] or the calcium-mobilizing second messenger InsP_3 [7,12,17]. It has been proposed that such long latencies argue against direct mechanisms such as the opening of calcium channels by conformational coupling, but are more consistent with models requiring the generation of messengers such as CIF [104,105]. However, these long latencies could also be explained by the fact that the entry component has been suppressed through the negative feedback effect of Ca^{2+} (Figure 1, pathway f). For example, in the exocrine pancreas the latent period preceding the onset of entry coincides with the time when the calcium concentration near the membrane is high, as reflected by the observation that the pump on the basal membrane is maximally stimulated [105]. Parallel Ca^{2+} measurements in *Xenopus* oocytes have revealed that the onset of the entry component coincided with the decline in the intracellular level of Ca^{2+} [7,17]. Further evidence that high intracellular levels of Ca^{2+} suppress entry were obtained by using flash photolysis of InsP_3 to produce a localized elevation of Ca^{2+} which then abolished the entry of Ca^{2+} [17]. It required up to 10 s for the entry mechanism to recover following inactivation through such a global elevation of calcium.

Very much shorter latencies have been recorded when the influx current is measured directly. In the case of Jurkat cells, flash photolysis of caged InsP_3 results in the onset of an inward current in less than 100 ms [11]. Similarly, the light-induced inward Ca^{2+} current in *Drosophila* photoreceptors has latencies as short as 7 ms [106]. It is difficult to envisage how these very rapid responses could occur by any of the proposed coupling mechanisms described earlier other than conformational coupling.

Oscillations in capacitative calcium entry

Oscillations in capacitative calcium entry can occur through two separate mechanisms. First, oscillations may arise indirectly by being entrained to a separate oscillator. Since many cells set up Ca^{2+} oscillations based on the periodic release of internal Ca^{2+} [22,107–109], entry might be switched on through the store depletion pathway (Figure 1, pathway g) each time the stores discharge their calcium. Measurements of entry during spiking, using the Mn^{2+} quench technique, have given conflicting results. In endothelial cells there was no evidence for oscillations in entry [94] but this was found to occur in synchrony with Ca^{2+} spikes in AR42J cells [110] and in Mardin–Darby canine kidney-focus cells [111]. The second mechanism is more direct in that the entry mechanism oscillates autonomously to set up periodic fluctuations in cytosolic calcium. Depletion of the intracellular stores with thapsigargin is particularly effective in setting up such oscillations in lymphocytes [112,113], parotid acinar cells [114,115] and HeLa cells [38].

There are two explanations for these oscillations in I_{CRAC}. First, the entry is driven by a periodic depletion and refilling of the stores [113]. An essential feature of this mechanism is that there has to be an intrinsic delay in order to explain why the influx of calcium is out of phase with the release of calcium from internal stores. The second explanation is that oscillations are set up by the periodic inactivation of I_{CRAC} through the inhibitory action of calcium (Figure 1, pathway f) [115].

Calcium entry transients

The way in which I_{CRAC} switches on displays some complex kinetics which reveal interesting properties relevant to the activation process. As described earlier, one of the difficulties of studying calcium entry (Figure 1, pathway a) is that it can be influenced by Ca^{2+} being released from internal stores (Figure 1, pathway b). One way of separating the two processes is to prevent entry, either by removing external calcium or by voltage-clamping the membrane at a low potential which prevents the CRAC channels from opening while the stores are being emptied using either InsP_3 or thapsigargin. Once the stores have been partially or completely depleted, entry can then be initiated by suddenly hyperpolarizing the membrane or by adding back the calcium [7,12,15,17,38]. Such experiments have revealed that entry is markedly biphasic, it switches on very quickly but then

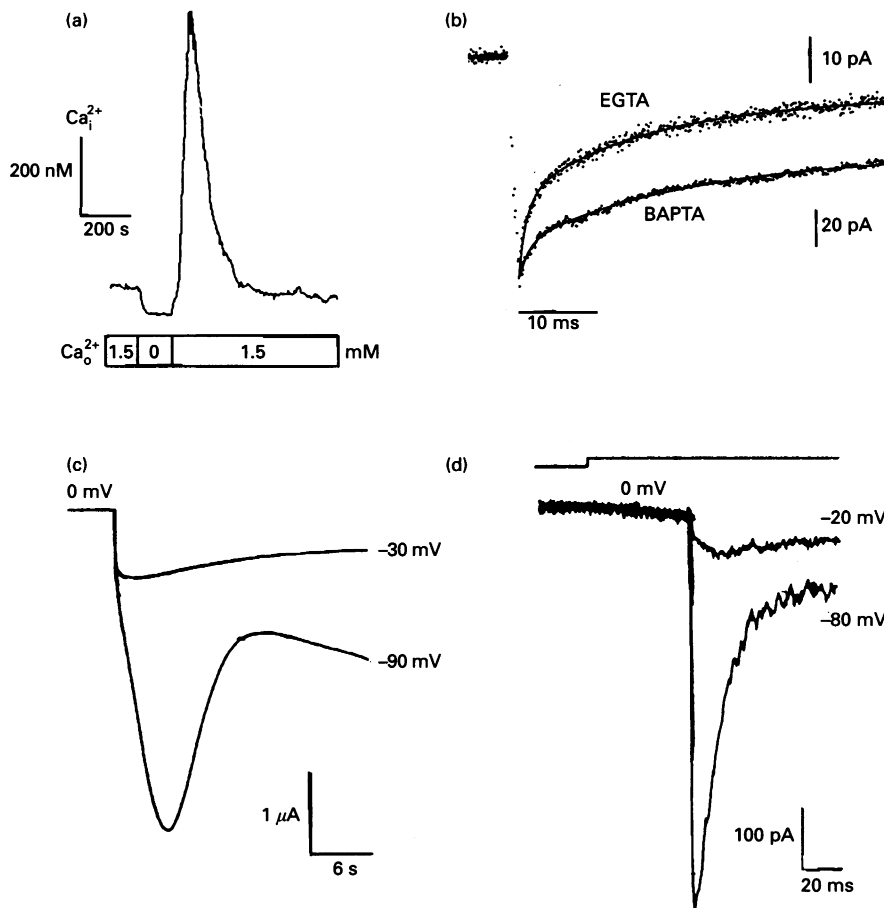

Figure 3 Kinetic aspects of capacitative calcium entry

(**a**) Ca^{2+} entry transient recorded from a single HeLa cell which had been pretreated with thapsigargin to activate capacitative Ca^{2+} entry {reproduced from Missiaen, L., De Smeldt, H., Parys, J. B., Oike, M. and Casteels, R. (1994) J. Biol. Chem. **269**, 5817–5823 [38] with the permission of the American Society for Biochemistry and Molecular Biology}. (**b**) Induction of I_{CRAC} by switching the membrane potential from 0 to −100 mV in a rat mast cell following depletion of the stores with 10 μM InsP_3 {reproduced from Hoth, M. and Penner, R. (1993) J. Physiol. (London), **465**, 359–386 [15] with the permission of the Physiological Society}. (**c**) Onset of capacitative Ca^{2+} entry in thapsigargin-treated *Xenopus* oocytes by switching the membrane potential from 0 to either −30 or −90 mV {reproduced from Petersen, C. C. H. and Berridge, M. J. (1994) J. Biol. Chem. **269**, 32246–32253 [12] with the permission of The American Society for Biochemistry and Molecular Biology}. (**d**) Inward currents induced in a *Drosophila* photoreceptor following a switch in membrane potential from 0 to either −20 or −80 mV. The entry mechanism was activated by low-intensity light as shown in the top trace {reproduced from Hardie, R. C. (1991) Proc. R. Soc. London Ser. B. **245**, 203–210 [27] with the permission of The Royal Society}.

reverses equally rapidly (Figure 3). The experiment on the HeLa cell is particularly informative because a pretreatment in zero calcium, which presumably removed the tonic Ca^{2+} inhibition on the entry mechanism, was necessary to display the large entry transient when external Ca^{2+} was reapplied (Figure 3a) [38]. A large Ca^{2+} overshoot was also observed in T lymphocytes following an incubation under conditions which prevented an influx of external Ca^{2+} [113]. Much faster kinetics are observed if the entry current is measured more directly and if membrane hyperpolarization is used to rapidly switch on I_{CRAC}. In rat mast cells, for example, switching the membrane potential from 0 to −100 mV resulted in an almost instantaneous inward current which then decayed by 64% with a half-time of about 10 ms (Figure 3b) [15]. This inactivation was considerably attenuated if the fast buffer BAPTA was used instead of EGTA. A similar observation was used by Zweifach and Lewis [39] to estimate that the inactivation site was located close (3–4 nm) to the mouth of the CRAC channel.

In the case of *Xenopus* oocytes, the influx of Ca^{2+} was monitored indirectly by measuring the Ca^{2+}-activated chloride current (Figure 3c). All these biphasic responses can be explained by reference to the bell-shaped activation curve depicted in Figure 2. As the channel opens, the positive feedback effect (Figure 1, pathway e) will ensure a rapid activation of entry but, as the level of Ca^{2+} rises, the negative feedback mechanism (pathway f) cuts in and entry ceases [17]. Since the biphasic response can be observed in oocytes with empty stores, it is concluded that the biphasic response can be induced through positive and negative calcium feedback pathways activated sequentially by calcium entering through the CRAC channels (Figure 1, pathway a) independently of Ca^{2+} release through InsP_3 receptors (pathway b in Figure 1).

Since Ca^{2+} seems to exert such a profound modulation of I_{CRAC}, it would be interesting to know what the activation kinetics are like when untrammelled by these feedback pathways. One way of addressing this issue is to use a charge carrier which fails to activate the feedback pathways. A good candidate is Ba^{2+} because it has no modulatory effects on the Ca^{2+}-sensitive sites on the InsP_3 receptor [36,116]. In the case of *Xenopus* oocytes, the use of Ba^{2+} has an additional advantage in that it fails to activate the chloride channels, thus allowing direct measurement of I_{CRAC} in the absence of the positive and negative feedback

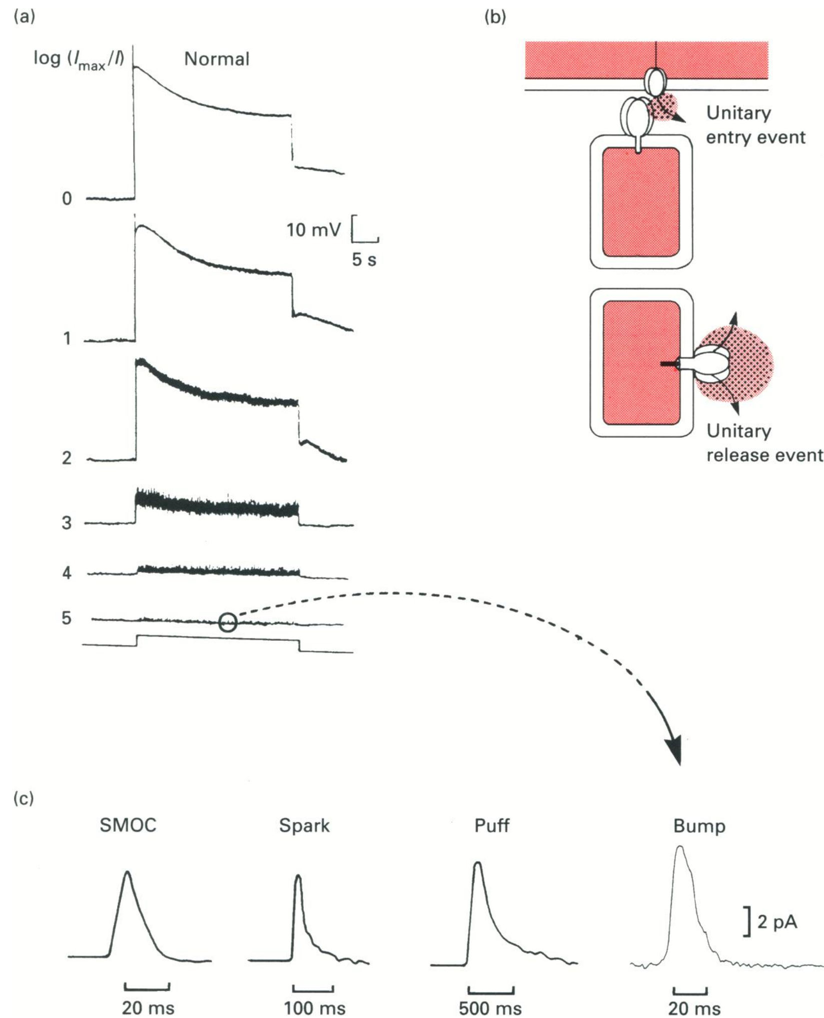

Figure 4 Spatial and temporal aspects of unitary Ca^{2+} signalling events

(**a**) Receptor potentials recorded from the photoreceptors of the blowfly *Lucilia* in response to increasing light intensities {reproduced from Barash, S., Suss, E., Stavenga, D. G., Rubinstein, C. T., Selinger, Z. and Minke, B. (1988) J. Gen. Physiol. **92**, 307–330 [119] by copyright permission of The Rockefeller University Press}. (**b**) Diagrammatic representation of unitary events associated with either entry or release. (**c**) Four examples of unitary events revealing similar kinetic properties. Spontaneous miniature outward current (SMOC) recorded from a sympathetic neuron {reproduced from Satin, L. S. and Adams, P. R. (1987) Brain Res., **401**, 331–339 [123] with the permission of Elsevier Science B. V.}; spark recorded from a cardiomyocyte {reprinted with permission from Cheng, H., Lederer, W. J. and Cannell, M. B. (1993) Science, **262**, 740–744 [124]. Copyright 1993 American Association for the Advancement of Science}; puff recorded from a *Xenopus* oocyte {reproduced from Yao, Y., Choi, J. and Parker, I. (1995) J. Physiol. (London), **482**, 533–553 [126] with the permission of The Physiological Society} and a bump recorded from a *Drosophila* photoreceptor (kindly supplied by Dr. R. C. Hardie).

effects activated by Ca^{2+} [7]. This inward Ba^{2+} current switched on quickly ($t_{\frac{1}{2}}$ 200 ms) but there was no evidence for a regenerative process nor was there any inactivation even after 20 s. Similarly, inactivation was greatly reduced in Jurkat T-lymphocytes when Ba^{2+} was the charge carrier [39]. The inactivation seen when Ca^{2+} is the charge carrier can thus be explained through the feedback effect on the InsP_3 receptor (Figure 1).

Drosophila photoreceptors

Drosophila photoreceptors offer a unique opportunity to study the process of Ca^{2+} entry through a mechanism which has properties similar to those associated with capacitative Ca^{2+} entry [31,32]. In response to light there is an increase in InsP_3 which then activates a light-induced inward Ca^{2+} current (LIC) resulting in the depolarization that constitutes the light response [117]. Molecular genetics has begun to dissect out the components of this signalling pathway [31,32,118]. The no receptor potential (norpA) mutants are incapable of generating a light response because of a defective PLC. As described earlier, the transient receptor potential (*trp*) mutants, which can initiate a light response but fail to maintain the plateau response, suggest a defect in a protein that resembles the CRAC channel [30,32]. It has been proposed that these photoreceptors may have a capacitative entry system [29,32] based on conformational coupling [31,32]. The LIC, which provides a direct measure of Ca^{2+} entry, has kinetics remarkably similar to those just described for the *Xenopus* oocytes (cf. Figures 3c and 3d). The biphasic response depends upon Ca^{2+} exerting first a positive and then a negative feedback effect on the entry process [27]. At high light intensities, an early rapid increase is soon reversed by negative feedback mechanisms before the entry process settles down to an equilibrium position determined by the level of light intensity [30] (Figures 3d and 4a).

Since these photoreceptors possess a transduction machinery specialized to provide high sensitivity (i.e. they can detect single photons), it is possible to study the operation of a few or perhaps just one of the ensembles shown in Figure 1. At very low light intensities, these receptors respond to single photons by generating unitary events known as bumps (Figure 4) [31,119]. The relationship between stimulus intensity and bump frequency, as described in *Lucilia* (Figure 4) [119] and *Drosophila* [120] photoreceptors, provides an interesting example of how Ca^{2+} entry can be graded depending on stimulus intensity. As light intensity increases, with the concomitant increase in the level of InsP_3, bump frequency goes up such that these unitary events begin to fuse together to form a noisy trace which then results in the onset of the global depolarization which constitutes the light response described earlier (Figure 4a).

The specific suggestion, therefore, is that at very low light intensities the arrival of a single photon activates a single rhodopsin/PLC complex to provide a localized elevation of InsP_3, which then activates either an individual or a collection of entry complexes to give the unitary bumps. Each bump has a characteristic shape, it has a rapid rising phase followed by a somewhat slower recovery phase which decays as a single exponential (Figure 4c). The width of the bump at half its maximum value is approx. 20 ms. Unitary Ca^{2+} signalling events with kinetics very similar to these bumps in *Drosophila* photoreceptors have been recorded in other cells (Figure 4c). Examples include the quantum emission domains (QED) in presynaptic terminals [121,122], the spontaneous miniature outward current (SMOC) in sympathetic neurons [123], the sparks in cardiac cells [124], bumps in *Limulus* photoreceptors [125] and the puffs in *Xenopus* oocytes [126]. All these events depend upon the brief opening of either a single or a small localized group of Ca^{2+} channels located either in the plasma membrane or on the stores (Figure 4b) to produce a localized cloud of calcium that builds up quickly and then decays away exponentially by passive diffusion as soon as the channel closes (Figure 4c). Note that the bumps observed in invertebrate photoreceptors have different origins. In *Limulus* they represent Ca^{2+} release from internal stores [125], whereas in *Drosophila* they seem to result from the conformational-coupling complexes responsible for calcium entry.

The main difference between bumps in *Drosophila* photoreceptors and the other unitary phenomena depicted in Figure 4(c) (e.g. SMOCs, sparks and puffs) is that the former depends upon external Ca^{2+} and may thus represent a unitary event of entry rather than of release (Figure 4b). This conclusion seems to be supported by recent measurements of intracellular Ca^{2+} concentration in *Drosophila* receptors responding to light [76,77]. Light induced a rapid increase in Ca^{2+} concentration which was spatially localized to the junction between the rhabdomeres and the cell body [77] where the calcium-storing submicrovillar cisternae are located [89]. When Ca^{2+} was removed from the outside medium, there was no elevation in calcium even though the membrane response remained much the same except that the activation and inactivation kinetics were greatly slowed down. In the absence of external calcium, the entry channels conduct Na^{+}, thus maintaining the normal depolarizing light-activated response. The implication of all this is that InsP_3 can continue to operate the entry mechanism without appearing to release its internal store of calcium. There are a number of explanations for this observation. One possibility is that the recording system may not have been sensitive enough to detect the minute amounts of calcium being released from the rather small stores found in *Drosophila* photoreceptors [31]. This seems unlikely because the system was sensitive enough to detect the Ca^{2+} entering through the surface channels which have conductances very much smaller than the large InsP_3-sensitive channels. An alternative explanation is that the InsP_3 receptor of *Drosophila* photoreceptors might be coupled to separate entry process which may be distinguished by whether or not they require store depletion [31,40]. As described earlier, *trp* requires store depletion but *trpl* does not [43]. This means that the light response in the wild-type fly might be a composite of two separate channel types acting in sequence, i.e. an initial response independent of store depletion (e.g. channels such as *trpl*) followed by channels activated by store depletion (e.g. *trp*). The initial response seen in *trp* mutants could then be explained by the operation of the first component. In summary, the properties of these *Drosophila* channels suggest that conformational coupling might be used to activate calcium entry independently of store depletion. Such a possibility leads on to the question concerning the calcium conductance of the InsP_3 receptor when it is part of a conformational coupling complex.

As discussed earlier, there is an interesting possibility that when the InsP_3 receptors are coupled to surface channels in a conformational-coupling mode (as in Figure 1) they lose their ability to gate calcium, i.e. the change in conformation is directed towards the opening of the surface channel (Figure 1, pathway a) instead of the channel in the ER. Some support for such a disengagement is apparent in the Ca^{2+}-free studies which results in the entry channels remaining open for very much longer, suggesting the absence of the negative feedback pathway operated by calcium [27,76,77]. If the InsP_3 receptor were capable of gating Ca^{2+}, it seems likely that it would have switched on the inhibitory loop. Removing external Ca^{2+} has effectively removed the Ca^{2+} feedback loops (pathways f and e in Figure 1) and thus resembles the experiment in *Xenopus* oocytes (described earlier) where Ba^{2+} replaced Ca^{2+} as a charge carrier. In both cases, the effect turns out to be the same, i.e. the entry pathway remains open for very much longer. The important conclusion to emerge from these studies in *Drosophila* is that InsP_3 might be capable of activating entry without necessarily stimulating release.

Conclusion

Capacitative calcium entry across the plasma membrane plays an essential role in calcium signalling. In some cells, such as lymphocytes and *Drosophila* photoreceptors, it can generate signals directly, whereas in other cells it serves to replenish the internal stores especially under conditions where these stores are generating repetitive calcium spikes. A number of models have been proposed to explain how the internal stores can regulate the CRAC channels in the plasma membrane. Some models propose the existence of diffusible messengers whereas others consider that information may be transferred more directly through a protein–protein interaction. On the basis of available information, it is difficult to decide between these different proposals. Considerable emphasis has been placed on the conformational coupling model since it is able to explain many features of capacitative calcium entry.

The properties of calcium entry through CRAC channels turn out to be remarkably similar in a number of different systems, thus suggesting a common mechanism. Of particular note is that this mechanism may have been adapted to produce the very rapid light response in *Drosophila* photoreceptors. It has been instructive, therefore, to compare this light response with the capacitative calcium entry found in other cells. The role of calcium as a feedback regulator of entry has been well-characterized in photoreceptors and may be a general feature determining the rapid activation and inactivation kinetics that

characterize capacitative calcium entry. The conformational coupling model considers that some calcium sensitivity may reside on the InsP_3 receptor, which is thought to be responsible for transmitting information from the ER to the plasma membrane. If the InsP_3 receptor is directly involved in mediating entry it may explain the mode of action of other activators and modulators. For example, the work on photoreceptors has suggested that InsP_3 might be able to activate entry directly. In addition, G-proteins and protein phosphorylation may exert their effects on capacitative calcium entry by modulating the proposed interaction between the InsP_3 receptor and the CRAC channel.

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