The Journal of Experimental Biology 200, 303–314 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB0598

INTRACELLULAR Ca^{2+} SIGNALLING IN SECRETORY CELLS

TREVOR J. SHUTTLEWORTH*

Department of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA

Summary

The secretion of ions and fluid plays a critical role in a variety of physiological activities that are vital to homeostatic mechanisms in animals. Control of such secretory activity is achieved by a range of neurotransmitters and hormones many of which act intracellularly by generating the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) and increasing cytosolic free calcium ion concentrations ($[Ca^{2+}]_i$). These increases are achieved by a combination of the $InsP_3$-induced release of Ca^{2+} from specific intracellular stores and the activation of Ca^{2+} entry from the extracellular environment. The $[Ca^{2+}]_i$ signal represents a balance between the adequate activation of components of the secretory mechanism and the avoidance of $[Ca^{2+}]_i$ levels that are toxic to the cell. Resting $[Ca^{2+}]_i$ is maintained low by the action of Ca^{2+} pumps on the intracellular stores and plasma membrane, with the result that gradients for Ca^{2+} movement into the cytosol from either of these two sources are very large and there is considerable potential for achieving rapid increases in $[Ca^{2+}]_i$. Consequently, for successful Ca^{2+} signalling, it is imperative that these two mechanisms of raising $[Ca^{2+}]_i$ (i.e. Ca^{2+} release and Ca^{2+} entry) are closely integrated. Current models emphasize the activation of Ca^{2+} entry as a downstream result of the emptying of the intracellular stores ('capacitative' model). Whilst this may be true for situations of maximal stimulation, recent experiments on the oscillatory $[Ca^{2+}]_i$ responses typical of more physiological levels of stimulation indicate a previously unsuspected, independent activation of Ca^{2+} entry involving arachidonic acid.

This arachidonic-acid-activated entry plays a key role, along with $InsP_3$, in inducing the repetitive release of Ca^{2+} from the stores to produce the $[Ca^{2+}]_i$ oscillations. In this way, the two components responsible for the elevation of $[Ca^{2+}]_i$ are intimately related and their dual effects closely coordinated, resulting in the finely tuned control of agonist-induced changes in $[Ca^{2+}]_i$.

Key words: exocrine cells, avian nasal gland, ion channels, Ca^{2+} oscillations, Ca^{2+} entry, inositol trisphosphate, arachidonic acid, phospholipase A_2.

Introduction

At first sight, it may seem that the secretion of ions and fluid is a rather specific and narrowly specialized activity that has little impact on overall homeostasis of the body. However, further consideration reveals that this activity underlies the functioning of a wide variety of activities that are vital to key homeostatic processes. These include digestion, respiration, reproduction, body-fluid balance and the sensing of the physical and chemical composition of the external environment. The fact that the simple disruption of the control of ion and fluid secretion underlies such devastating human diseases as cholera and cystic fibrosis serves to illustrate dramatically the critical role this process plays in our lives.

Given the demonstrated importance of ion and fluid secretion in homeostatic processes, and the diverse roles that it plays in the body, it is not surprising that this activity is under the control of a wide range of different neurotransmitters and hormones. Although it is clear that these regulators of secretory activity can operate intracellularly *via* many different signalling systems, this review will focus on only one of these systems – the system that involves the generation of inositol phosphates and increases in the cytosolic concentrations of free calcium ions ($[Ca^{2+}]_i$). The reasons for this emphasis are threefold. First, this particular system is probably the one most widely applicable to the various cell types involved in ion and fluid secretion, and is certainly the most intensely studied to date. Second, historically speaking, studies on a variety of secretory cells and tissues have played a major role in the development of much of our current understanding of this intracellular signalling system. For example, the very earliest studies showing that activation of receptors for certain agonists (specifically muscarinic cholinergic agonists) leads to an increase in the turnover of inositol-containing membrane phospholipids were carried out on cells from the avian salt-secreting nasal gland (Hokin and Hokin, 1964). The first direct confirmation of the causal association between inositol and the generation of Ca^{2+} signals, originally proposed by Michell (1975), came from work on the blowfly salivary gland (Berridge and Fain, 1979), as did the first indication of the

*e-mail: tshut@pharmacol.rochester.edu.

oscillatory nature of these responses (Rapp and Berridge, 1981). The watershed discovery of $InsP_3$ as the all-important link between the agonist-activated turnover of phosphoinositides, which takes place in the plasma membrane, and the mobilization of Ca^{2+} from stores in the cytosol was performed on pancreatic acinar cells (Streb *et al.* 1983), and much of our current thinking about the nature and control of agonist-activated Ca^{2+} entry in non-excitable cells comes from studies on lacrimal and parotid exocrine cells (Putney, 1990). Finally, from a homeostatic viewpoint, it is clear that this signalling system is also the one that poses the most problems for the cell (see below).

Mechanism of secretion

Despite the diverse roles played by the secretion of ions and fluid in different tissues, the basic mechanism involved shows remarkable consistency. Essentially the process is one of a secondary-active transepithelial transport of chloride (Frizzell *et al.* 1979). In this process, a basolateral Na^+ pump (Na^+/K^+ P-ATPase) generates a gradient for the entry of Na^+ from the extracellular medium across the basolateral membrane. The entry of Na^+ down this gradient is coupled to the uphill movement of Cl^- into the cell *via* a $Na^+/2Cl^-/K^+$ symporter (cotransporter) that is sensitive to inhibition by the so-called loop diuretics, such as bumetanide and furosemide. The operation of this basolateral symporter results in the accumulation of Cl^- in the cell to levels significantly in excess of the electrochemical equilibrium. Under appropriate conditions, apical Cl^- channels are opened, allowing the exit of Cl^- to the apical surface. At the same time, the opening of basolateral K^+ channels permits the recycling of this ion across the basolateral membrane. The increase in K^+ conductance serves to maintain the cell in a hyperpolarized state *vis-a-vis* the Cl^- equilibrium potential, thereby maintaining the driving force for the apical exit of Cl^-. In this way, Cl^- is transported from the basal to the apical side of the epithelium, generating an electrical gradient (apical negative). This gradient drives the secretion of Na^+ *via* a paracellular route (through the tight junctions) between the cells. The net secretion of both Na^+ and Cl^- causes water to follow osmotically, resulting in the secretion of fluid. Although the opening of apical Cl^- channels and basolateral K^+ channels is the primary event in the initiation of secretion, other processes are activated to sustain the response. For example, the loss of both Cl^- and K^+ from the cell that is associated with the initiation of secretion leads to cell shrinkage as water follows osmotically. It is this reduction in cell volume and/or the fall in cytosolic Cl^- concentration that activates the basolateral symporter to maintain the secretory process (Lytle and Forbush, 1996).

Although this basic scheme is applicable to many secretory tissues, some important variations are found. For example, in certain exocrine cells, a basolateral Cl^-/HCO_3^- antiporter (exchanger), usually coupled with a parallel Na^+/H^+ antiporter, either assists or replaces the $Na^+/2Cl^-/K^+$ symporter for the uptake of Cl^-. In other, more highly specialized tissues, the basic plan is even further modified and other transporters are involved such as the apical Cl^-/HCO_3^- antiporter in the HCO_3^--secreting cells of the pancreatic ducts and the apical K^+/H^+ P-ATPase found in the acid-secreting parietal cells of the stomach. A rather different system is found in certain insect secretory epithelia (e.g. lepidopteran midgut and Malpighian tubules), as well as in specific renal epithelia in vertebrates, where the transport of a variety of ions is secondarily coupled to gradients generated by an apical proton-translocating V-ATPase (Harvey, 1992; Wieczorek, 1992).

The calcium signal

As noted above, many different receptors on the surface of secretory cells exert their effects on the initiation and control of cell function *via* pathways involving the elevation of $[Ca^{2+}]_i$. It is now known that these increases are achieved by a combination of the $InsP_3$-induced release of Ca^{2+} from specific intracellular stores and the activation of Ca^{2+} entry from the extracellular medium. In recent years, considerable advances have been made in detailing the processes involved, particularly the generation of $InsP_3$ and its role in the release of Ca^{2+} from specific intracellular stores. The generation of $InsP_3$ results from the hydrolysis of an inositol-containing membrane phospholipid, phosphatidylinositol 1,4,5-bisphosphate, in a reaction catalyzed by a receptor-activated phosphoinositide-specific phospholipase C (Berridge and Irvine, 1989; Berridge, 1993). The resulting products are the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ is water-soluble and diffuses into the cytosol where it binds to specific receptors on certain intracellular Ca^{2+} stores, resulting in the mobilization of Ca^{2+} from these stores. The precise nature of the agonist-sensitive intracellular Ca^{2+} stores is unclear, other than the fact that they represent some component of the endoplasmic reticulum (Pozzan *et al.* 1994). In contrast, considerable information now exists regarding the nature of the $InsP_3$ receptors and their functioning (Mikoshiba, 1993; Shuttleworth, 1994*a*; Taylor and Traynor, 1995). It is known that the $InsP_3$ receptor protein contains both $InsP_3$-binding and Ca^{2+}-release properties, so the receptor can be considered as an example of an *intracellular* ligand-gated ion channel (Ferris *et al.* 1989; Mayrleitner *et al.* 1991). Isolation, cloning and sequencing of the receptor has shown it to consist of a tetramer of approximately 260 kDa subunits. The $InsP_3$-binding domain resides at the extreme N-terminal region (last 400 residues) of the protein, whilst the C-terminal portion contains six putative membrane-spanning domains that make up, or at least contain, the Ca^{2+} channel (Mignery and Südhof, 1990). Binding of $InsP_3$ induces a large conformational change in the protein (Mignery and Südhof, 1990), which presumably underlies the transduction process responsible for opening of the Ca^{2+}-channel portion. The region between the $InsP_3$-binding domain and the putative Ca^{2+}-channel domain constitutes the so-called 'coupling' domain, some 1400–1500 amino acids in length, which lies in the cytoplasm of the cell. It is now clear that this coupling

domain is the site of a variety of modulatory actions of potential functional significance, including phosphorylation by protein kinase A (Supattapone *et al.* 1988). Studies of the conduction properties of the isolated receptor reconstituted in lipid bilayers indicate that the channel is a rather non-specific cation-selective channel (Bezprozvanny and Ehrlich, 1994) with a single-channel conductance of 20–26 pS with 50–53 mmol l^{-1} Ca^{2+} as the current carrier (Maeda *et al.* 1991; Bezprozvanny *et al.* 1991; Watras *et al.* 1991). Addition of $InsP_3$ induces complex channel openings with four conductance states that appear as multiples of the basic 20 pS state, with level 3 (60 pS) predominating. Recent studies have revealed that at least three (possibly as many as five) separate genes can encode distinct $InsP_3$ receptor subtypes. The three subtypes whose distribution is most widespread and whose full sequence is known (subtypes 1–3) show significant sequence diversity, particularly in the Ca^{2+}-channel region of the protein and in the so-called coupling domain (Yamamoto-Hino *et al.* 1994). Such structural diversity raises the possibility of functional implications – an idea that has assumed increased significance with the demonstration of the specific tissue distribution of receptor subtypes and of unique patterns of subtype expression during differentiation (Fujino *et al.* 1995; Sugiyama *et al.* 1994). However, the true physiological implications of this subtype-specific expression remain unclear.

Clearly, although many important details remain to be described, recent years have seen considerable advances in our understanding of the $InsP_3$ receptor and its role in Ca^{2+} release. However, because the intracellular stores only contain a finite amount of Ca^{2+}, the activity of various mechanisms removing Ca^{2+} from the cytosol (e.g. Ca^{2+} transporters on the plasma membrane and other organelles) will mean that such release can only result in a transient increase in $[Ca^{2+}]_i$. Sustained $[Ca^{2+}]_i$ signals are entirely dependent on an agonist-activated Ca^{2+} entry and, as will be discussed below, far less is known about this component of the overall $[Ca^{2+}]_i$ signal. Despite the uncertainties in the underlying mechanisms, the net result of these two components, Ca^{2+} release and Ca^{2+} entry, is the classic biphasic $[Ca^{2+}]_i$ signal – an initial rapid rise, largely caused by the $InsP_3$-dependent release of Ca^{2+} from the stores, followed by a decline to a new sustained elevated $[Ca^{2+}]_i$ 'plateau', which is entirely dependent on the enhanced entry of extracellular Ca^{2+}.

The role that these changes in $[Ca^{2+}]_i$ play in the control of ion and fluid secretion has also been extensively studied and is now fairly clear (Petersen, 1992). Apical Cl^- channels and basolateral K^+ channels sensitive to increases in $[Ca^{2+}]_i$ have been described from a variety of different secretory cell types, and it is the opening of these channels that is responsible for the initiation of secretory activity (see above). For example, in secretory cells from the avian nasal gland, the Ca^{2+}-activated K^+ channels are of the so-called 'maxi-K^+' type – large-conductance, Ca^{2+}- and voltage-dependent channels inhibitable by tetraethylammonium (TEA^+) and by charybdotoxin (Martin and Shuttleworth, 1994*a*; Wu *et al.* 1996). The Ca^{2+}-activated Cl^- channels are kinetically and pharmacologically distinct from the cyclic-AMP-activated Cl^- channels which are of the so-called 'cystic fibrosis transmembrane conductance regulator' (CFTR) type, although both co-exist within the same cell (Martin *et al.* 1994).

Spatial and temporal features of Ca^{2+} signals

In recent years, techniques that allowed changes in intracellular $[Ca^{2+}]$ to be studied within individual cells have revealed that $[Ca^{2+}]_i$ signals in non-excitable cells are far more complex and subtle than the simple elevation of $[Ca^{2+}]_i$ to a new sustained level as was originally supposed. These complex responses include repetitive oscillations or waves of $[Ca^{2+}]_i$ (Berridge and Irvine, 1989; Berridge, 1990; Lechleiter *et al.* 1991; Rooney and Thomas, 1993). Increases in agonist concentration generally increase the frequency of these oscillations, but with little change in their amplitude, suggesting that the original continuously graded (analog) signal of the hormone or neurotransmitter is converted intracellularly into a frequency-encoded (digital) signal. Yet another manifestation of the spatial and temporal subtlety of $[Ca^{2+}]_i$ signals that applies particularly to polarized secretory cells is the presence of highly localized so-called 'trigger zones' in cells where agonist-induced changes in $[Ca^{2+}]_i$ occur without affecting other regions of the cytosol (Kasai and Augustine, 1990; Toescu *et al.* 1992). The potential physiological implications of such trigger zones are fairly clear in that they will permit the targetted activation of appropriate effectors within specific areas in the cell. Overall, the current consensus is that these more complex responses, which are characteristically produced by low agonist concentrations, represent the true physiologically relevant signals as they probably reflect the normal situation in the intact tissue. As a consequence, it has become increasingly clear that the specific spatial and temporal patterns of $[Ca^{2+}]_i$ signalling in exocrine, as well as other, cells have a major impact on the effective control of cell activity.

Several different models for $[Ca^{2+}]_i$ oscillations have been proposed, including those in which the concentration of $InsP_3$ oscillates (Meyer and Stryer, 1991; Cuthbertson and Chay, 1991) and those in which $InsP_3$ concentrations are constant and the oscillations result from the repetitive Ca^{2+} release from the $InsP_3$-sensitive stores (De Young and Keizer, 1992; Atri *et al.* 1993) or *via* a Ca^{2+}-induced Ca^{2+} release (CICR) from $InsP_3$-insensitive stores (Goldbeter *et al.* 1990). Although components of each of these models may apply to the responses in different cell types, it is clear that a requirement for oscillating concentrations of $InsP_3$ is not applicable to the oscillatory responses of many cells where, for example, oscillations can be produced by the introduction of non-hydrolyzable $InsP_3$ analogs (Wakui *et al.* 1989). Similarly, a requirement for $InsP_3$-insensitive pools of releasable Ca^{2+}, such as those sensitive to ryanodine and caffeine, is far from absolute as oscillations are commonly seen in cells where such stores are absent (e.g. avian nasal gland cells; Martin and

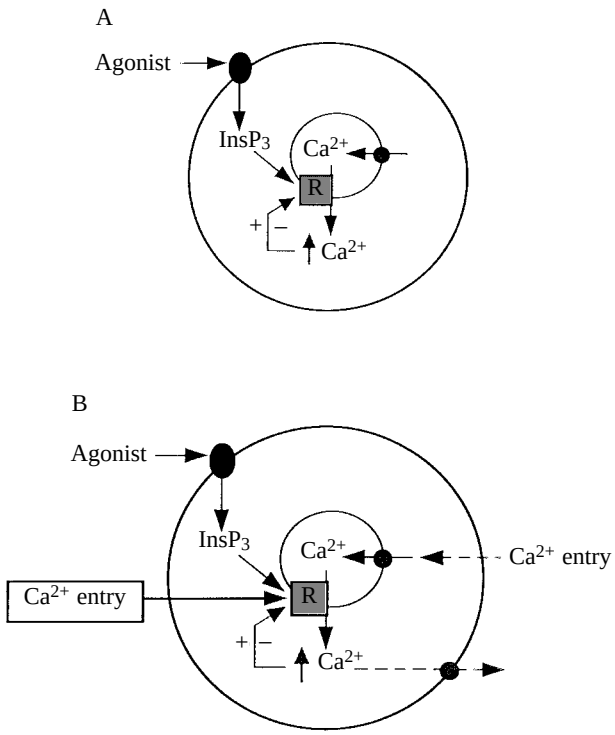

Fig. 1. Diagram illustrating the critical role of Ca^{2+} entry in the control of $[Ca^{2+}]_i$ oscillations. (A) The 'minimal' model for inositol trisphosphate ($InsP_3$)-dependent $[Ca^{2+}]_i$ oscillations where the intrinsic oscillatory mechanism results from the biphasic effect of released Ca^{2+} on the $InsP_3$ receptor (R; see text for details). (B) The critical role of Ca^{2+} entry in controlling and modulating this oscillatory mechanism lies not in the recharging of the stores between each oscillation, but in its action on the $InsP_3$-sensitized $InsP_3$ receptors to trigger the release of Ca^{2+}.

Shuttleworth, 1994*b*). Although the details vary, the 'minimal' models for such examples rely on the unique kinetics of the $InsP_3$ receptor and its sensitivity to $[Ca^{2+}]_i$ (Fig. 1A). Increases in cytosolic $[Ca^{2+}]$ have a biphasic effect on $InsP_3$-induced Ca^{2+} release – initially enhancing Ca^{2+} release and subsequently decreasing it (Iino, 1990; Finch *et al.* 1991). This behavior is duplicated by the isolated receptor incorporated into lipid bilayers. Here, the curve for the Ca^{2+}-dependency of channel open probability in the presence of $InsP_3$ is 'bell-shaped', with a peak at approximately 250–300 nmol l^{-1} Ca^{2+} (Bezprozvanny *et al.* 1991). The consequence is that, initially, Ca^{2+} released as a result of the action of $InsP_3$ will exert a positive feedback on further Ca^{2+} release. Then, as cytosolic $[Ca^{2+}]$ rises further (particularly in the region close to the open $InsP_3$ receptor), it will have a negative feedback action, effectively closing the channel and allowing the stores to refill. This cycle can then be repeated to result in a train of oscillations in $[Ca^{2+}]_i$. Computer models of varying complexity based on such biphasic behavior can successfully simulate the different characteristics of the oscillatory $[Ca^{2+}]_i$ responses observed in a variety of cells with reasonable accuracy (De Young and Keizer, 1992; Atri *et al.* 1993).

The precise basis for the biphasic effects of Ca^{2+} on the $InsP_3$ receptor that are critical to such models is unclear. The possible involvement of phosphorylation–dephosphorylation events has been suggested (Zhang *et al.* 1993) but this cannot account for all the data, particularly those obtained with the purified receptor. One possibility is that the biphasic Ca^{2+} effect may be related to the finding that increases in $[Ca^{2+}]$ reversibly switch the receptor to a non-conducting (or poorly conducting) configuration with a high affinity for $InsP_3$ (Pietri *et al.* 1990). There is considerable evidence for such Ca^{2+}-dependent conformational switches affecting binding affinity (see, for example, Marshall and Taylor, 1994), but it remains to be directly demonstrated that such changes in binding affinity are associated with changes in the Ca^{2+} conductance of the release channel. The important point is that this property of the $InsP_3$ receptor makes its activity sensitive to the levels of both $InsP_3$ and Ca^{2+} in the cytosol, and $InsP_3$ and Ca^{2+} therefore act as co-agonists at the receptor (Finch *et al.* 1991). Furthermore, because the positive feedback of increases in $[Ca^{2+}]_i$ on the channel are induced more rapidly than the subsequent negative feedback effects, the Ca^{2+}-releasing properties of the $InsP_3$ receptor are also influenced by the *rate* at which cytosolic $[Ca^{2+}]$ is changing (Taylor and Traynor, 1995).

The role of Ca^{2+} entry in Ca^{2+} signalling

As pointed out above, the sustained, elevated 'plateau' of $[Ca^{2+}]_i$ typically seen at high agonist concentrations is entirely dependent on Ca^{2+} entry. As such, it is readily abolished by removal of extracellular Ca^{2+} or by maneuvers known to interfere with Ca^{2+} entry, such as blockage of the entry pathway by a range of trivalent and divalent metal ions (e.g. La^{3+}, Cd^{2+}), by the drug SK&F 96563 or simply by reducing the driving force for Ca^{2+} entry by depolarizing the cell membrane potential. The role of Ca^{2+} entry in such responses is therefore clear. In contrast, the role of Ca^{2+} entry in the more complex and subtle types of response referred to above has received only scant attention. Indeed, examination of the basic models for $[Ca^{2+}]_i$ oscillations reveal that, in each case, the underlying mechanism is essentially intrinsic to the cell and there is no absolute requirement for any Ca^{2+} entry. Such a notion is supported by the ability of at least some cell types to continue oscillating in the absence of extracellular Ca^{2+}. Nevertheless, to a greater or lesser degree, Ca^{2+} oscillations in most cells are ultimately sensitive to the removal of extracellular Ca^{2+}. The variability seen probably reflects differences in the activities of the transporters removing cytosolic Ca^{2+} at the plasma membrane and in the stores, and/or simply differences in the surface to volume ratios of different cell types. In this context, it is worth pointing out that some caution should be used in the interpretation of experiments demonstrating the effects, or lack thereof, of extracellular Ca^{2+} on oscillations where the oscillations are recorded as changes in Ca^{2+}-activated currents using whole-cell patch-clamp techniques. This protocol necessitates that the

experimenter impose their own extrinsic level of cytosolic Ca^{2+} buffering, determined by the composition of the solution in the pipette, and this may or may not match the intrinsic buffering of the intact cell. That this can have dramatic effects on the apparent sensitivity of agonist-induced oscillations to extracellular Ca^{2+} is shown by data obtained in avian nasal gland cells (Martin and Shuttleworth, 1994*b*). Here, the sensitivity of oscillations in Ca^{2+}-activated currents to extracellular Ca^{2+} in cells in a perforated-patch configuration, where the intrinsic cytosolic Ca^{2+} buffering is maintained, was markedly different from that in the same cells under standard whole-cell patch-clamp conditions. Similarly, measurements of acetylcholine-induced oscillations in pancreatic acinar cells using Fura-2 indicate an acute sensitivity to extracellular Ca^{2+} (Yule and Gallacher, 1988), whilst measurements from the same cells using whole-cell patch-clamp techniques report that oscillations in Ca^{2+}-dependent currents are, at least initially, unaffected by removal of extracellular Ca^{2+} (Wakui *et al.* 1989).

In any event, even in those cells where $[Ca^{2+}]_i$ oscillations demonstrate little or no absolute dependency on extracellular Ca^{2+}, Ca^{2+} entry has been shown to exert a modulating influence on such responses, principally by affecting oscillation frequency (Kawanishi *et al.* 1989; Girard and Clapham, 1993). Such effects are usually assumed to result from a role of Ca^{2+} entry in replenishing intracellular Ca^{2+} lost during the oscillations and in recharging the intracellular stores (Rink and Hallam, 1989; Berridge, 1993). It was hypothesized that the interspike interval represented the time required for the stores to refill to some set point before they could be discharged by the agonist-induced levels of $InsP_3$ (Berridge, 1993, 1994; Thorn, 1995). Reducing Ca^{2+} entry therefore slowed the rate at which this refilling took place, lengthening the interspike interval and reducing oscillation frequency. In experiments directly testing this hypothesis (Shuttleworth and Thompson, 1996*a*), we found that, under conditions designed progressively to deplete oscillating stores by modestly reducing Ca^{2+} entry, the oscillations continued until the stores were completely empty. Clearly, the intrinsic ability of the stores to oscillate was not dependent on their degree of loading. Furthermore, in oscillating cells, the stores apparently refill completely during the downstroke from the peak of each oscillation and are already recharged by the beginning of the interspike interval (Shuttleworth and Thompson, 1996*a*). The interspike interval cannot, therefore, reflect the period required to refill the oscillating stores. These findings are inconsistent with the hypothesis that the critical role of Ca^{2+} entry during oscillations is to recharge the oscillating stores during the interspike interval. Indeed, recent reports have indicated that the relative activity of the organellar Ca^{2+} pumps on the stores is much higher than that of the plasma membrane Ca^{2+} pumps (Camello *et al.* 1996). Such activity would favor Ca^{2+} recycling between the cytosol and the stores during oscillations, with relatively little being lost to the extracellular environment and, hence, little requirement for Ca^{2+} entry to recharge the stores between each spike.

If the demonstrated role of Ca^{2+} entry in modulating $[Ca^{2+}]_i$ oscillations is not related to the refilling of the stores between each oscillation, then it follows that it must be involved in regulating the *emptying* of the oscillating stores. Consistent with this hypothesis, in avian nasal gland cells, the acute inhibition of Ca^{2+} entry in the continued presence of agonist (e.g. by addition of La^{3+} or SK&F 96365 or by depolarizing the cell in a high-K^+ medium) results in the immediate cessation of the oscillations (Martin and Shuttleworth, 1994*b*). Importantly, this occurs without the measurable depletion of the agonist-sensitive Ca^{2+} pool. These data reveal two important findings. First, they show that, at low agonist concentrations (i.e. under the very conditions where oscillations are most commonly observed), agonist-generated levels of $InsP_3$ are in themselves inadequate to result in a regenerative $[Ca^{2+}]_i$ signal. Second, they show that a component of Ca^{2+} entry is critically required to drive the generation of such signals, apparently by inducing the repetitive release of Ca^{2+} from internal stores. This role of Ca^{2+} entry in the triggering of $InsP_3$-dependent Ca^{2+} release (Fig. 1B) probably reflects its action as a co-agonist, with $InsP_3$, at the $InsP_3$ receptors (Finch *et al.* 1991). This effect may be restricted to stores close to the plasma membrane whose discharge then provides additional Ca^{2+} to induce the $InsP_3$-dependent release at the remaining agonist-sensitive stores.

The nature of Ca^{2+} entry during Ca^{2+} signals

On the basis of the above discussions, it would seem that Ca^{2+} entry plays a critical role in essentially all $[Ca^{2+}]_i$ signals whether in the form of a sustained 'plateau' response or in the form of oscillations. However, studies on the nature of Ca^{2+} entry have almost exclusively concerned the type of responses where sustained elevated $[Ca^{2+}]_i$ signals are produced and agonist-sensitive intracellular Ca^{2+} stores undergo extensive and prolonged depletion (e.g. at high agonist concentrations). Despite some continued controversy, current ideas concerning the activation of Ca^{2+} entry under these conditions have focused on the so-called 'capacitative' model (Putney, 1986, 1990) in which the enhanced entry of Ca^{2+} results solely from the emptying of the agonist-sensitive intracellular Ca^{2+} stores, without any *direct* requirement for inositol phosphates. Evidence in support of such a mechanism in a wide variety of different cell types is extensive. More recently, direct electrophysiological measurements of a Ca^{2+}-selective current that is activated by depletion of the intracellular Ca^{2+} stores (so-called I_{CRAC}) have been made (Hoth and Penner, 1992, 1993; Penner *et al.* 1993), and its selectivity and activation/inactivation properties characterized (Hoth, 1995; Zweifach and Lewis, 1995*a*,*b*). However, the key question as to the nature of the signal responsible for relaying the status of the intracellular stores to the Ca^{2+} entry pathway in the plasma membrane remains unresolved. Several possible 'candidate' messengers have been suggested, including cyclic GMP (Bahnson *et al.* 1993; Xu *et al.* 1994), a member of the 'small

G-protein' family (Fasolato *et al.* 1993; Bird and Putney, 1993) or a currently poorly characterized 'Ca^{2+} influx factor' (CIF) (Randriamampita and Tsien, 1993).

An intriguing variation on this model has been proposed in which the Ca^{2+} status of the stores is transmitted to the plasma membrane Ca^{2+} entry pathways (I_{CRAC} channels) *via* direct protein–protein interactions (Berridge, 1995). In this 'conformational coupling' model, it is envisaged that the large cytoplasmic domain of the $InsP_3$ receptor is responsible for relaying information on the store status directly to the entry channels. Implicit in such a model is the requirement that the $InsP_3$ receptor be able to sense and respond to changes in the Ca^{2+} content of the store. Unfortunately, studies examining possible influences of the store content on $InsP_3$ receptor function have produced contradictory results, and this is currently an area of considerable controversy (Shuttleworth, 1995). In addition, for a conformational coupling mechanism to operate, the stores involved must lie in close proximity to the Ca^{2+} entry channels in the plasma membrane. This limitation has led to an additional important requirement for the model. It is known that the Ca^{2+} entry pathway demonstrates a marked Ca^{2+}-induced inactivation, and a similar inactivation has been recorded directly in the depletion-activated I_{CRAC} channels (Zweifach and Lewis, 1995*a*,*b*). Given this inactivation, it would seem that the $InsP_3$ receptors involved in conformational coupling must not be involved in Ca^{2+} release as the resulting high local concentrations of Ca^{2+} would inactivate the entry pathway (Berridge, 1995). The suggestion has been made that both the localization at the plasma membrane and the inhibition of the Ca^{2+} release activity may involve the binding of ankyrin to the receptor (Berridge, 1995). However, the sequence identified as the proposed ankyrin-binding site (amino acids 2546–2556 in the mouse, 2548–2558 in the rat; Bourguignon and Jin, 1995) actually lies between membrane-spanning domains 5 and 6 in most models of the $InsP_3$ receptor sequence (Yamamoto-Hino *et al.* 1994). This would place this putative binding site at the luminal face of the receptor, a location which is incompatible with the roles proposed. In conclusion, it would seem that, although the conformational coupling model can explain much of the data regarding the activation of Ca^{2+} entry by depletion of the stores, some significant problems remain and, in the absence of any direct evidence, its validity remains uncertain.

As already discussed, the kinds of responses that typify stimulation with more physiological levels of agonists, such as $[Ca^{2+}]_i$ oscillations and $[Ca^{2+}]_i$ waves, involve only the transient emptying of the intracellular stores. As Ca^{2+} entry was generally thought to be of little importance in these responses, it had been largely ignored. The first indication that agonist-activated Ca^{2+} entry under these conditions may not conform to the capacitative model came from studies where the quenching of intracellular Indo-1 by Mn^{2+} was used to evaluate the rate of Ca^{2+} entry during oscillatory responses. In avian nasal gland cells, the quenching rate of intracellular Indo-1 by Mn^{2+} is enhanced some three- to sixfold during $[Ca^{2+}]_i$ oscillations, but the rate of quenching remains constant during the alternate emptying and refilling of the stores (Martin and Shuttleworth, 1994*b*; Shuttleworth and Thompson, 1996*b*). Similar findings have been reported in endothelial cells (Jacob, 1990) and HeLa cells (Thorn, 1995). Of course, the precise relationships between Mn^{2+} quenching and agonist-activated Ca^{2+} entry are unclear, and such results must therefore be interpreted with caution. Nevertheless, the finding of a non-oscillating Mn^{2+} quench in cells whose stores are alternately emptying and refilling does suggest that the rate of at least one component of agonist-activated Ca^{2+} entry is unaffected by the status of the oscillating agonist-sensitive Ca^{2+} stores. As such, this is inconsistent with capacitative models.

More substantial evidence for a non-capacitative Ca^{2+} entry during oscillations came from experiments on cells where the agonist-induced oscillations had been stopped by the acute inhibition of Ca^{2+} entry upon depolarization of the cell (Shuttleworth and Thompson, 1996*b*). As discussed above, because Ca^{2+} entry is critically required to trigger the emptying of the stores (Martin and Shuttleworth, 1994*b*), acute inhibition of Ca^{2+} entry stops the oscillations but does so without any measurable depletion of the stores, despite the continued presence of agonist. Significantly, returning such cells to conditions where Ca^{2+} entry can occur (i.e. repolarization) results in the immediate restoration of $[Ca^{2+}]_i$ oscillations whose size, shape and frequency are identical to those seen immediately prior to depolarization. As Ca^{2+} entry is known to be an absolute requirement to drive these oscillations (see above), their immediate restart upon restoration of the driving force for entry implies that the Ca^{2+} entry pathway must have remained activated under conditions where it is known that the agonist-sensitive stores are not depleted. Such data are clearly difficult to reconcile with current versions of the capacitative model.

In addition, because it is inherent in the capacitative model that the activation of Ca^{2+} entry must be preceded by the emptying of the stores, it follows that inhibition of Ca^{2+} entry should be without effect on the *initiation* of a $[Ca^{2+}]_i$ response. Examination of this proposition under conditions where oscillatory responses would be expected (i.e. low agonist concentrations) revealed that reduction of Ca^{2+} entry, by depolarization of the cell by exposure to a high-K^+ medium, significantly increased the time delay before the first $[Ca^{2+}]_i$ spike. More dramatically, inhibition of Ca^{2+} entry by brief pre-exposure of the cells to SK&F 96365 completely blocked the initiation of an oscillatory response on addition of agonist. Oscillations rapidly begin, however, on subsequent washout of the SK&F 96365 (Shuttleworth and Thompson, 1996*b*). If, as these data suggest, agonist-activated Ca^{2+} entry at these agonist concentrations is critically involved in the *initial* release of Ca^{2+} from intracellular stores, then such entry cannot be gated by any mechanism dependent on depletion of the stores. Hence, such entry cannot be *via* a capacitative mechanism, at least as currently understood. Finally, we attempted a more direct evaluation of the Ca^{2+} entry pathway under conditions of $[Ca^{2+}]_i$ oscillations. A basic paradigm of the capacitative

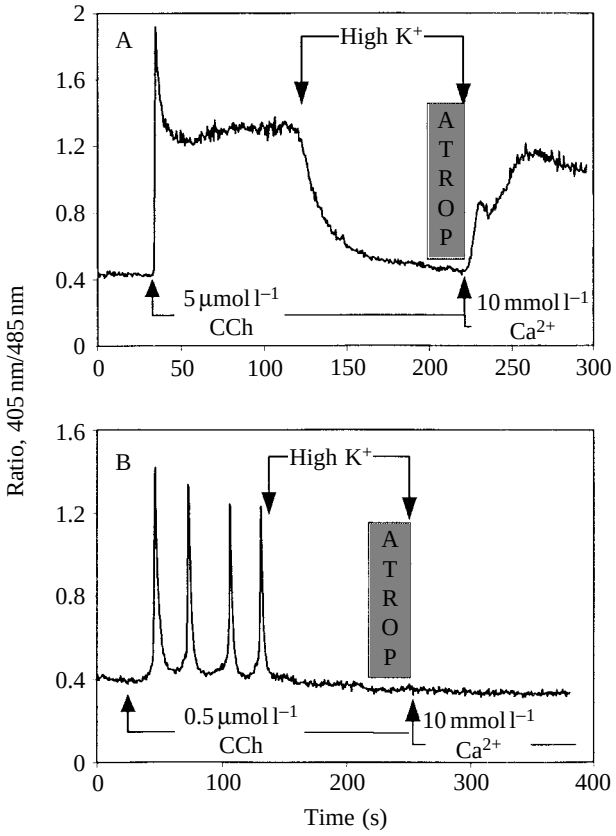

Fig. 2. Demonstration of capacitative Ca^{2+} entry in a cell showing a sustained $[Ca^{2+}]_i$ signal (A) and its absence in a cell showing an oscillating $[Ca^{2+}]_i$ response (B). Traces reflect the changes in $[Ca^{2+}]_i$ determined as the ratio of the emitted fluorescence at 405 nm and 485 nm in individual Indo-1-loaded cells excited at 350 nm measured using a photon-counting microfluorimeter system mounted on an inverted microscope. Cells were first stimulated either with 5 µmol l^{-1} carbachol (CCh) to induce a sustained elevation in $[Ca^{2+}]_i$ (A) or with 0.5 µmol l^{-1} carbachol to induce oscillations in $[Ca^{2+}]_i$ (B). Subsequently, Ca^{2+} entry was inhibited by depolarizing the cells using a high-K^+ medium ($[K^+]$=70 mmol l^{-1}), followed by addition of atropine (indicated by the block marked ATROP, 5 or 0.5 µmol l^{-1} as appropriate). This displaces the carbachol from its receptor and allows $InsP_3$ levels to return to resting values (complete within less than 15 s). However, refilling of agonist-depleted stores is prevented by the continued presence of the high-K^+ medium. 30 s after the addition of atropine, the medium was changed to one containing 10 mmol l^{-1} Ca^{2+} to reveal any enhanced Ca^{2+} entry remaining. By definition, such entry would correspond to a capacitative entry as it would be dependent only on the depleted state of the agonist-sensitive stores and not on the sustained activation of the receptor or on the presence of elevated levels of $InsP_3$. Data redrawn from Shuttleworth and Thompson (1996*b*).

model is that, following depletion of the stores by exposure to an agonist, Ca^{2+} entry remains activated even after removal of the agonist and the decay of elevated levels of inositol phosphates, *as long as refilling of the stores is prevented*. Such a response can readily be demonstrated in nasal gland cells when treated with agonist concentrations sufficient to produce a sustained elevation in $[Ca^{2+}]_i$ (Fig. 2A). However, experiments utilizing an exactly parallel protocol in oscillating cells failed to reveal any such response (Fig. 2B). The protocol employed also effectively ruled out the possibility of Ca^{2+} entry being gated in a capacitative manner but by only a subset of the total agonist-sensitive stores, perhaps those lying closest to the plasma membrane, with only the remaining stores undergoing the cyclical emptying and refilling associated with oscillations. In this case, the sustained depletion of such a subset of the stores should still give rise to a capacitative Ca^{2+} entry, but no such entry could be detected in these experiments.

To summarize, a variety of different experiments have produced findings that are inconsistent with the proposal that the observed agonist-activated Ca^{2+} entry during oscillations is occurring *via* a capacitative mechanism. Possible alternative mechanisms for Ca^{2+} entry under these circumstances fall into two groups – either entry is being controlled by some component of the PLC/inositol phosphate pathway [such as *via* an action of $InsP_3$ and/or inositol tetrakisphosphate ($InsP_4$) at the plasma membrane] or the agonist is activating a distinct signalling pathway that is independently responsible for the control of Ca^{2+} entry. Our efforts to try and resolve this question have recently revealed the presence of a novel signalling mechanism for the activation of Ca^{2+} entry under conditions of low agonist concentrations that is quite distinct from the 'classical' capacitative mechanism. This mechanism involves the receptor-activation of a phospholipase A_2 and the subsequent generation of arachidonic acid (Shuttleworth, 1996). Using cells from the avian nasal gland, we found that activation of muscarinic receptors stimulated a marked release of arachidonic acid in cells preloaded with [^{3}H]arachidonic acid. Furthermore, this response was unaffected by preloading the cells with the Ca^{2+} chelator BAPTA at a concentration sufficient to obliterate completely any agonist-induced changes in $[Ca^{2+}]_i$, showing that the observed agonist-induced activation of arachidonic acid release was not merely a downstream effect of the simultaneous $[Ca^{2+}]_i$ signal. It was further shown that the simple addition of low concentrations (2–5 µmol l^{-1}) of exogenous arachidonic acid to otherwise unstimulated cells was sufficient to result in an increase in Ca^{2+} entry.

Additional evidence came from experiments using the biscoclaurine (bisbenzylisoquinoline) alkaloid isotetrandrine. Although this compound has been shown to produce many different effects in various cell types (see references in Akiba *et al.* 1992), these diverse actions can generally be ascribed, at least at the low micromolar concentrations employed, either to effects on various voltage-operated Ca^{2+} channels (specifically by interacting at the benzothiazipine binding site) (Felix *et al.* 1992) or to effects on the activation of a phospholipase A_2 (PLA_2) and the consequent generation of arachidonic acid (AA) (Akiba *et al.* 1992; Hashizume *et al.* 1991). For example, isotetrandrine has been shown to inhibit amylase release and the $[Ca^{2+}]_i$ oscillations induced by agonists acting at the high-affinity cholecystokinin (CCK) receptor in pancreatic acinar cells (Tsunoda and Owyang, 1995) – actions that are believed

to involve PLA_2 and arachidonic acid. In avian nasal gland cells, we found that isotetrandrine potently and reversibly blocked $[Ca^{2+}]_i$ oscillations induced by low agonist concentrations, and did so by inhibiting the associated non-capacitative entry of Ca^{2+}, an effect confirmed by Mn^{2+} quench studies (Shuttleworth, 1996). This effect clearly did not result from an action on voltage-operated Ca^{2+} channels as these are absent in avian nasal gland cells, and the classic benzothiazipine receptor antagonist diltiazem was completely without effect on $[Ca^{2+}]_i$ oscillations. However, consistent with a proposed action in inhibiting a PLA_2, isotetrandrine significantly inhibited the observed receptor-mediated release of arachidonic acid. It did not, however, have any effect on the receptor-mediated generation of $InsP_3$. As noted above, isotetrandrine potently blocked the non-capacitative Ca^{2+} entry associated with low agonist concentrations. Further experiments showed that this was a specific effect as isotetrandrine was completely without effect on the sustained $[Ca^{2+}]_i$ signals seen at high agonist concentrations, signals that are known to reflect a capacitative Ca^{2+} entry. It also failed to affect the capacitative entry activated following depletion of the stores with the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor thapsigargin. A similar failure to affect agonist-induced or thapsigargin-induced capacitative Ca^{2+} entry in lacrimal and parotid cells has previously been reported for the related compound tetrandrine (Rossier *et al.* 1993). As a final confirmation of the role of a PLA_2/AA pathway in the activation of the non-capacitative Ca^{2+} entry, it was shown that the inhibition of $[Ca^{2+}]_i$ oscillations by isotetrandrine could be reversed by addition of low concentrations of exogenous arachidonic acid as well as by addition of a small peptide that is part of a recently isolated PLA_2-activating protein (PLAP-peptide) (Clark *et al.* 1991).

The conclusion from these studies is that the agonist-enhanced, non-capacitative Ca^{2+} entry seen during $[Ca^{2+}]_i$ oscillations results from the generation of AA as a result of a receptor-mediated activation of a PLA_2 (Shuttleworth, 1996). The observed effects appear to involve an action of arachidonic acid itself as inhibitors of the cyclo-oxygenase and lipoxygenase pathways for arachidonic acid metabolism failed to mimic the effects of isotetrandrine on $[Ca^{2+}]_i$ oscillations. In fact, lipoxygenase inhibition changed an oscillatory $[Ca^{2+}]_i$ response into a sustained plateau response, an effect that presumably reflects the accumulation of arachidonic acid following inhibition of its metabolism. The precise mechanism by which the PLA_2 is activated is, as yet, unclear. For example, it may involve the carbachol-induced activation of a distinct PLA_2-coupled G-protein. However, as argued above, our data show that the continued oscillatory signal requires the *combined* action of increases in $[InsP_3]$ and Ca^{2+} entry. We know that the activation of phospholipase C (PLC) in the nasal gland cells involves a G_q-type G-protein (Hildebrandt and Shuttleworth, 1993), and one way in which such a combined response could be coordinated would be for both actions to be mediated *via* the same G-protein, with the $G\alpha$ subunit activating PLC and the $G\beta\gamma$ subunits activating a PLA_2. Significantly, activation of a PLA_2 by $G\beta\gamma$ subunits has been suggested in other systems (Jelsema and Axelrod, 1987; Kim *et al.* 1989; Tsunoda and Owyang, 1995).

Implications for cellular Ca^{2+} homeostasis

What then are the homeostatic implications of these various phenomena for the cell? It is clear that, in successful Ca^{2+} signalling, a finely tuned balance between the adequate activation of components of the secretory mechanism (e.g. K^+ and Cl^- channels) and the avoidance of $[Ca^{2+}]_i$ levels that are toxic to the cell is achieved. These toxic effects are multifarious and include the inappropriate activation of Ca^{2+}-dependent proteases, phosphatases, kinases, phospholipases and endonucleases, as well as effects on the organization and function of cytoskeletal elements such as microtubule polymerization and microfilament formation (Nicotera *et al.* 1992). In this context, much has been made of the 'homeostatic' advantages of oscillations *versus* sustained $[Ca^{2+}]_i$ signals. Such advantages include an improved signal-to-noise ratio, the avoidance of desensitization or accommodation of the target effector, the possibility of utilizing different components of the multiple coding information inherent in oscillations, and the avoidance of excessive Ca^{2+} pumping resulting from sustained elevations in $[Ca^{2+}]_i$. On this basis, the claim that Ca^{2+} signalling at the cellular level may have evolved towards a frequency-modulated mode of action for reasons of economy and safety – both of which are undoubtedly sound homeostatic principles – may have some validity. However, in such discussions, no discrimination is generally made regarding the source or origin of the respective changes in $[Ca^{2+}]_i$ and how the mechanisms responsible for producing the changes in $[Ca^{2+}]_i$ are carefully controlled to operate in a 'homeostatically responsible' manner.

The magnitude of this problem for the cell should not be overlooked. In this review, I have attempted to show that all Ca^{2+} signals in non-excitable cells ultimately involve components of both intracellular Ca^{2+} release and extracellular Ca^{2+} entry. The electrochemical gradients driving Ca^{2+} into the cytosol *via* either of these processes are extremely large. Whilst the magnitude of these gradients is part of what makes cytosolic Ca^{2+} such an excellent rapid signalling mechanism, it does carry the danger of producing excessive increases in $[Ca^{2+}]_i$. Given the requirement for careful control over the changes in $[Ca^{2+}]_i$ associated with successful Ca^{2+} signalling and the avoidance of the lethal effects of high $[Ca^{2+}]_i$ discussed above, it is clearly imperative that these two mechanisms of raising $[Ca^{2+}]_i$ (i.e. release and entry) be closely integrated. This review has presented evidence for the presence of two distinct mechanisms for the activation of Ca^{2+} entry by agonists in non-excitable cells – the well-known capacitative mechanism that is dependent on the depletion of agonist-sensitive intracellular Ca^{2+} stores (Putney, 1990; Berridge, 1995) and a newly described non-capacitative mechanism involving the activation of a PLA_2 and the generation of

arachidonic acid (Shuttleworth, 1996). Why are there two forms of Ca^{2+} entry activation, what are their individual properties and how do these properties influence and indicate their respective roles in the cell?

As discussed, current models for agonist-activated Ca^{2+} entry emphasize the capacitative model where, under normal circumstances, the activation of Ca^{2+} entry is a result of the $InsP_3$-mediated emptying of the intracellular stores (Putney, 1990). The obvious implication of this sequential mechanism is that depletion of the stores must precede the activation of Ca^{2+} entry. Although, as already mentioned, the details of the mechanisms involved in this sequence of events are far from clear, there is evidence that it incurs a significant delay in the activation process. For example, in avian nasal gland cells, the activation of Mn^{2+} quenching is delayed by some 20–30 s following the peak of store discharge at supramaximal agonist concentrations (Shuttleworth, 1994*b*). Similar delays have been found in several other cell types. More direct measurements of the depletion-dependent I_{CRAC} have indicated that the onset of activation occurs after a delay of approximately 5–6 s and is followed by a rather slow exponential increase to maximal levels with a time constant of some 18–27 s (Hoth and Penner, 1993). Such slow activation may indicate the involvement of a biochemical sequence of events in the transmission of the information on the status of the stores to the entry channels in the plasma membrane (Shuttleworth, 1994*b*). Alternatively, it could result from the effects of a Ca^{2+}-dependent inactivation mechanism that has been demonstrated in I_{CRAC} channels (Zweifach and Lewis, 1995*a*,*b*), although the activation time-course appears to be unaffected by the presence of high concentrations of the Ca^{2+}-chelators BAPTA or EGTA in the cell (Hoth and Penner, 1993). Whatever its origin, such slow activation kinetics are clearly not optimal for the fine control of a $[Ca^{2+}]_i$ response, particularly during the transient emptying and refilling of the stores associated with oscillations. Furthermore, at least in some cell types, the steady-state quantitative relationship between store depletion and capacitative Ca^{2+} entry is markedly non-linear, with the result that the stores must be 30–40 % depleted before entry begins to be significantly enhanced (Alvarez *et al.* 1994). Overall, given the limitations imposed by these specific properties of the capacitative mechanism, it would seem to be neither very sensitive nor subtle in its response and therefore is not well-adapted for achieving a finely tuned $[Ca^{2+}]_i$ signal accurately reflecting agonist action at the plasma membrane receptor. However, the capacitative mechanism is well-suited for maintaining the capacity of the stores – i.e. keeping them recharged with Ca^{2+}. This is obviously necessary for the cell to be able to respond to the next signal. In addition, there is now extensive evidence that the intrastore Ca^{2+} has a critical influence on a range of other cell functions, including protein folding and trafficking as well as cell division and proliferation (Waldron *et al.* 1994). Perhaps this maintenance of the overall, long-term status of the intracellular Ca^{2+} stores is the main function of the capacitative mechanism in cells.

In contrast to the sustained 'plateau' $[Ca^{2+}]_i$ responses seen with maximal stimulation, it seems clear that the main source of the Ca^{2+} involved in the oscillatory $[Ca^{2+}]_i$ signals typical of physiological levels of stimulation is the intracellular agonist-sensitive stores. This is to be expected given that the Ca^{2+} pump activity on these stores (SERCA pump) is 2–14 times higher than the plasma membrane Ca^{2+} pump activity in the physiological range of $[Ca^{2+}]_i$ (Camello *et al.* 1996). Indeed, there would appear to be sound energetic reasons why it is advantageous for Ca^{2+} signals to be generated principally by release and re-uptake from the stores (Taylor, 1995). If the main source of the Ca^{2+} involved in oscillations is the stores, then it follows that the point at which fine control of the oscillations is achieved must be the site of Ca^{2+} release – i.e. the $InsP_3$ receptor. Indeed, it seems that the properties of the $InsP_3$ receptor are uniquely suited for such a role. As already discussed, the operation of the $InsP_3$-mediated Ca^{2+} channel is controlled by the concentrations both of $InsP_3$ and of cytosolic Ca^{2+}, which act as co-agonists at the receptor, and also by the rate at which $[Ca^{2+}]_i$ is changing (Taylor and Traynor, 1995). It would seem, therefore, that the $InsP_3$ receptor is the ideal signal transducer for converting the agonist activation of a receptor in the plasma membrane into controlled changes in $[Ca^{2+}]_i$. These considerations lead to the concept of the $InsP_3$ receptor as '*the controller of* $[Ca^{2+}]_i$' rather than merely a simple conduit for Ca^{2+} release. As noted, the activity of the $InsP_3$-gated channel is controlled by the concentration of $InsP_3$, by the concentration of Ca^{2+} and by the rate of change in $[Ca^{2+}]_i$. For appropriately sensitive signalling and accurate transduction of the primary message, a system is needed whereby these three parameters can be independently modulated but, at the same time, all three should accurately reflect the degree of activation of the plasma membrane receptor by the agonist. It is difficult to envisage how these complex interactions could be achieved with the strictly sequential pathway inherent in the capacitative model. In contrast, it is precisely under these conditions that the separate, but coordinated, activation of $InsP_3$ generation and Ca^{2+} entry offered by the parallel PLC/$InsP_3$ and PLA_2/AA signalling pathways discussed above probably have their relevance (Fig. 3). We have shown that, under physiological conditions of stimulation, the agonist-generated levels of neither $InsP_3$ nor arachidonic acid are adequate on their own to induce a meaningful $[Ca^{2+}]_i$ signal. Only when both are activated together are $[Ca^{2+}]_i$ oscillations induced (Shuttleworth, 1996). The model proposed (Fig. 3) invokes the known co-agonist properties of $InsP_3$ and cytosolic Ca^{2+} on the $InsP_3$ receptor. Because of its rapid diffusion through the cytosol, it is likely that cytosolic concentrations of $InsP_3$ are essentially uniform during agonist stimulation (Allbritton *et al.* 1992). The role of the generated levels of $InsP_3$ is to sensitize the $InsP_3$ receptors on the stores such that a Ca^{2+} entry activated by arachidonic acid induces the opening of the Ca^{2+} release channels to initiate a Ca^{2+} signal. The probability of such a sequence of events occurring is therefore determined by the sustained level of $InsP_3$ which sensitizes the receptors and by the magnitude (and rate) of the local change in $[Ca^{2+}]_i$ produced by the AA-induced Ca^{2+} entry.

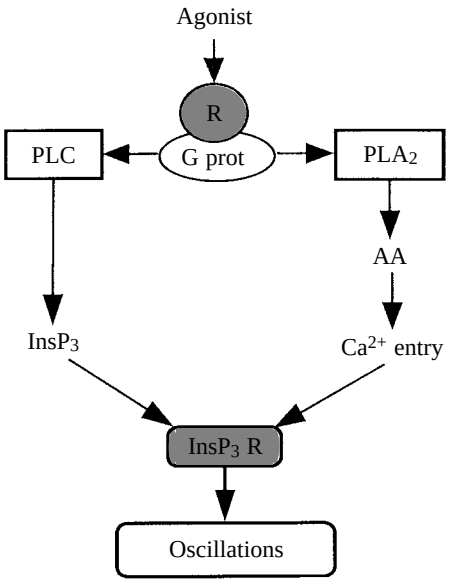

Fig. 3. Diagram illustrating the proposed dual parallel signalling pathways activated at low agonist concentrations that are involved in the initiation and control of oscillatory $[Ca^{2+}]_i$ signals. The phospholipase C/inositol trisphosphate (PLC/$InsP_3$) pathway acts to sensitize the $InsP_3$ receptors ($InsP_3R$) whilst the phospholipase A_2/arachidonic acid (PLA_2/AA) pathway, by activating a non-capacitative Ca^{2+} entry, repeatedly triggers the sensitized $InsP_3$ receptors to release stored Ca^{2+}. R, agonist receptor; G prot, G-protein(s).

Neither of these is effective alone, but both are independently related to the degree of agonist stimulation (Fig. 3). This primary event may be restricted to stores close to the plasma membrane, the $InsP_3$ receptors of which are more likely to be exposed to changes in $[Ca^{2+}]_i$ associated with Ca^{2+} entry. Once initiated, however, the Ca^{2+} pulse released from these stores will, if large enough, trigger release from other $InsP_3$-sensitized stores deeper in the cytoplasm. In this way, complex $[Ca^{2+}]_i$ signals in the form of spatially restricted 'trigger zones' or transient oscillations or waves that traverse the cytosol can be generated. In this model, the inherent repetitive oscillatory cycle is dependent on the biphasic influences of $[Ca^{2+}]_i$ on the $InsP_3$ receptor much as in the basic oscillation model originally discussed, but now both of the known required components of the $[Ca^{2+}]_i$ signal (Ca^{2+} release and Ca^{2+} entry), along with the levels of each of the co-agonists responsible for triggering this Ca^{2+} release ($InsP_3$ and cytosolic $[Ca^{2+}]$), are coupled separately, but in a co-ordinated manner, to levels of agonist stimulation. The mechanism provides the means whereby low-amplitude analog signals produced by low concentrations of agonist (namely, $InsP_3$ generation and AA-induced Ca^{2+} entry), when present together, are accurately converted into large-amplitude, but repetitively transient (i.e. frequency-encoded), signals by their combined actions at the $InsP_3$ receptor. In this way, the $InsP_3$ receptor acts as both an amplifier and an analog-to-digital converter, as well as a coincidence detector. If the sensitive and faithful transduction of relevant signals to appropriate effectors is a critical component of any successful homeostatic system, then this intracellular system is elegantly adapted for its role in the control of secretion.

Work from the author's laboratory included in this review was supported by NIH grant GM40457. The contributions of Dr J.-P. Hildebrandt and Dr S. C. Martin, and the excellent technical assistance of Jill L. Thompson, are gratefully acknowledged.

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