Inositol phosphates and cell signalling

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Inositol 1,4,5-trisphosphate is a second messenger which regulates intracellular calcium both by mobilizing calcium from internal stores and, perhaps indirectly, by stimulating calcium entry. In these actions it may function with its phosphorylated metabolite, inositol 1,3,4,5-tetrakisphosphate. The subtlety of calcium regulation by inositol phosphates is emphasized by recent studies that have revealed oscillations in calcium concentration which are perhaps part of a frequency-encoded second-messenger system.

KNOWLEDGE of the phosphoinositides and their role in cell signalling has expanded enormously since we reviewed this field five years ago[1]. Stimulation of cell-surface receptors initiates hydrolysis of a membrane-bound inositol lipid, which produces at least two second messengers—diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$). These messengers are generated by a membrane transduction process comprising three main components: a receptor, a coupling G protein and phosphoinositidase C (Figs 1 and 2). DAG acts by stimulating protein kinase C (ref. 2), whereas $Ins(1,4,5)P_3$ releases calcium from internal stores[1,3]. These pathways form the cornerstone of an ubiquitous transduction mechanism now known to regulate a large array of cellular processes including metabolism, secretion, contraction, neural activity and cell proliferation[1-7]. The second messenger role of DAG has been reviewed recently[2], and here we concentrate on the contribution of the inositol phosphates.

An enormous number of inositol phosphates have now been found in eukaryotic cells, and details of some of the metabolic pathways linking them have been elucidated[10-12] (summarized in Fig. 1; see panel on right for details of inositol phosphate numbering). A myriad of suspected or postulated functions have been ascribed to them; some of the more plausible are mentioned briefly below, but here we concentrate principally on the messenger functions of $Ins(1,4,5)P_3$ and related compounds, and particularly on their involvement in the generation of intracellular calcium signals. As more and more studies are made on single cells, it is becoming apparent that the phosphoinositide transduction system is highly organized in both space and time, and we present a spatiotemporal model that may help to unify the emerging ideas about intracellular calcium signalling.

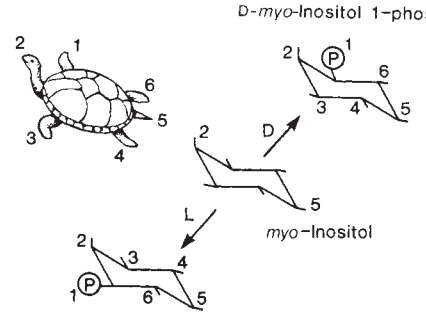
Multiple calcium pools

When cells respond to calcium-mobilizing agonists that act through the hydrolysis of inositol lipids, they draw upon both intracellular and extracellular sources of calcium. Although the latter remains constant, the size and distribution of the internal pools is highly variable. $Ins(1,4,5)P_3$ releases calcium from a non-mitochondrial pool which has characteristics which suggest that it is the endoplasmic reticulum (ER)[13], but only part of this pool seems to be $Ins(1,4,5)P_3$-sensitive; on average $Ins(1,4,5)P_3$ releases approximately 30–50% of the calcium taken up by the non-mitochondrial pool of calcium, the remainder of which can be released by calcium ionophores. This evidence implies that the $Ins(1,4,5)P_3$-insensitive pool is a separate membrane compartment which may have distinct calcium-pumping characteristics[14].

The anatomical location and identity of the $Ins(1,4,5)P_3$-sensitive and -insensitive pools are uncertain. Supattapone *et al.*[15] have purified a protein of relative molecular mass 260,000 (260K) that is thought to be the $Ins(1,4,5)P_3$ receptor. Immunocytochemical studies using a specific antibody on Purkinje cells (which have an extraordinarily high receptor density) reveal that this receptor is localized on the nuclear envelope and on parts of the ER, particularly near the nucleus[16]. Another candidate for the $Ins(1,4,5)P_3$-sensitive calcium pool, at least in peripheral tissues, is the calciosome, a small membrane vesicle which has some properties characteristic of the sarcoplasmic reticulum of muscle[17]. Calciosomes contain the calcium-binding protein calsequestrin and have calcium pumps with immunological properties resembling those in muscle. It is still not clear how calciosomes are related to the $Ins(1,4,5)P_3$-sensitive or -insensitive stores.

Stereochemistry and abbreviations of inositol phosphates

There has been much confusion in the literature concerning the nomenclature of the family of inositol phosphates. Strict adherence to the original IUPAC rules has added further confusion in that addition or removal of a phosphate could necessitate a switch between D- and L-systems of numbering. *myo*-Inositol, represented here in its chair conformation, has a plane of symmetry running through carbon atoms 2 and 5 thus dividing the molecule into chiral halves[8]. Agranoff's turtle is a useful mnemonic for remembering inositol phosphate numbering[9]. Substitution on carbon atoms 1,3,4 or 6 will alter this symmetry and the product must now be designated as D or L to define its position as illustrated for the D- and L-*myo*-inositol 1-phosphate enantiomers (which both occur naturally, see Fig. 1).



A new convention, recently published by the IUPAC, suggests that all inositol phosphates may be numbered as the D-*myo*-inositol derivative (abbreviated as Ins). Using this convention, the L-*myo*-inositol 1-phosphate (shown above) would adopt the numbers of the D-configuration and becomes D-*myo*-inositol 3-phosphate, abbreviated to Ins(3)*P*. The following are abbreviations for some of the more common inositol lipids and inositol polyphosphates appearing in the text:

Ins(4)P	D-*myo*-inositol 4-monophosphate
$Ins(1,4)P_2$	D-*myo*-inositol 1,4-bisphosphate
$Ins(1,4,5)P_3$	D-*myo*-inositol 1,4,5-trisphosphate
$Ins(1:2cyc,4,5)P_3$	D-*myo*-inositol (1:2-cyclic,4,5)-trisphosphate
$Ins(1,3,4,5)P_4$	D-*myo*-inositol 1,3,4,5-tetrakisphosphate
$InsP_5$	Inositol pentakisphosphate (unspecified isomer)
$InsP_6$	Inositol hexakisphosphate (phytic acid)
PtdIns	Phosphatidylinositol
PtdIns(4)P	Phosphatidylinositol 4-phosphate
$PtdIns(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate

Another question concerns whether or not the $Ins(1,4,5)P_3$-insensitive pool can contribute to calcium signals initiated by $Ins(1,4,5)P_3$. Studies on permeabilized cells and cell fractions have shown that GTP can release Ca^{2+} from both pools independently of $Ins(1,4,5)P_3$ (ref. 18) and that this release can be blocked by treatments that have no effect on the $Ins(1,4,5)P_3$-sensitive system. These properties closely resemble the GTP-dependent control of vesicular protein transport through the Golgi[19] and, with the relatively constant levels of GTP in cells, the physiological significance of GTP-induced calcium release in intact cells is doubtful. It has been suggested, however, that GTP may somehow enlarge the $Ins(1,4,5)P_3$-sensitive pool at

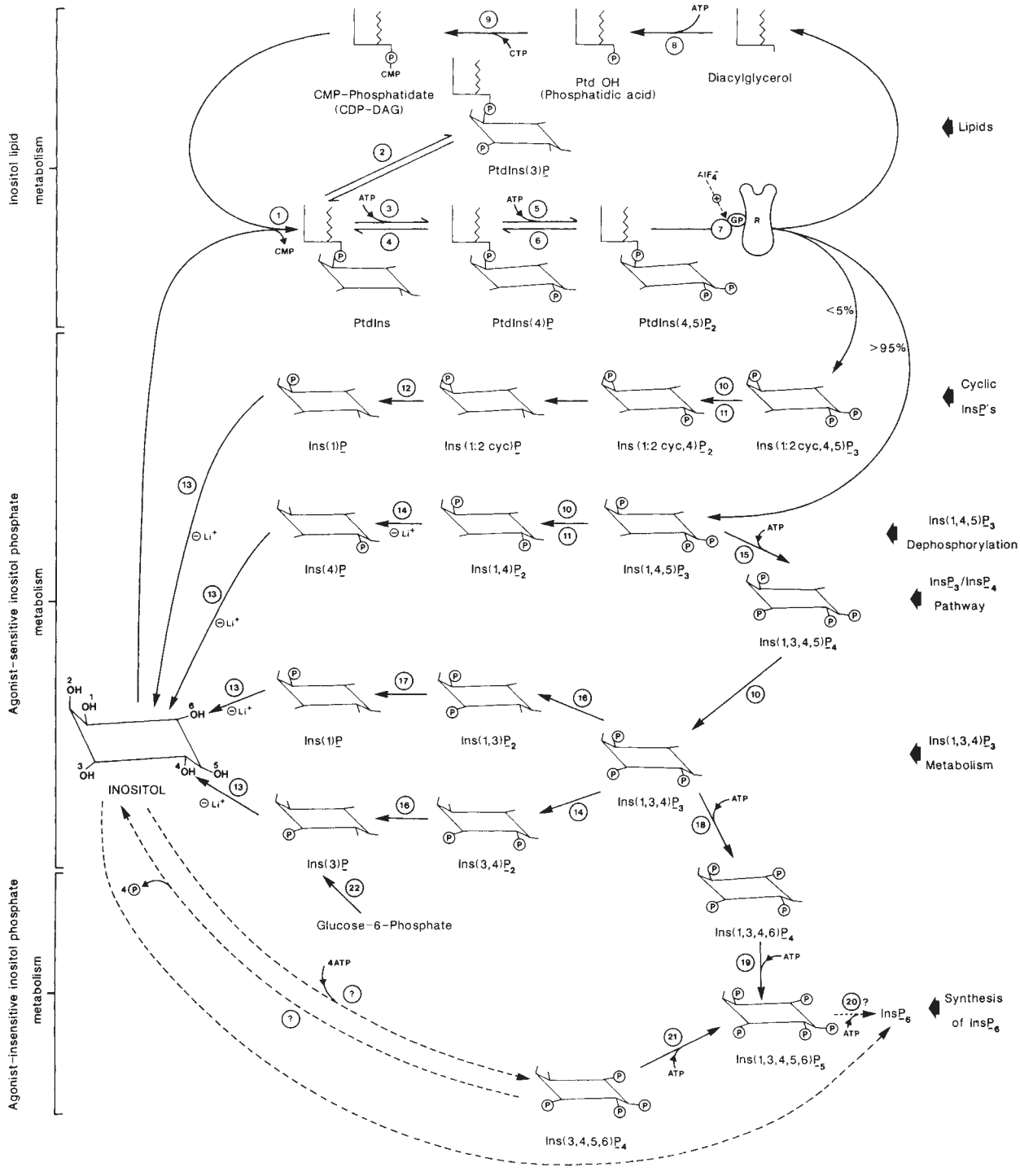


FIG. 1 Summary of known (solid arrows) and suspected (dashed arrows) routes of metabolism of compounds containing inositol and phosphate. To avoid further complexity, the phosphatidylinositol glycans are not illustrated. *myo*-Inositol is represented in its chair configuration and all the inositol phosphates are numbered in the D-isomer configuration (see panel on previous page). The enzymes are 1, PtdIns synthetase (CMP-PA; inositol phosphatidyltransferase); 2, PtdIns-3-kinase (type I); 3, PtdIns-4-kinase (type II); 4, PtdIns(4)P phosphomonoesterase; 5, PtdIns(4)P-5-kinase; 6, $PtdIns(4,5)P_2$ phosphomonoesterase; 7, phosphoinositidase C; 8, diacylglycerol kinase; 9, CMP-PA synthetase; 10, $Ins(1,4,5)P_3/Ins(1,3,4,5)P_4$-5-phosphatase; 11, $Ins(1,4,5)P_3$-5-phosphatase; 12, Ins(1:2cyc)P phosphodiesterase; 13, InsP phosphatase; 14, inositolpolyphosphate-1-phosphatase; 15, $Ins(1,4,5)P_3$-3-kinase; 16, inositolpolyphosphate-4-phosphatase; 17, $Ins(1,3)P_2$-3-phosphatase; 18, $Ins(1,3,4)P_3$-6-kinase; 19, $Ins(1,3,4,6)P_4$-5-kinase; 20, $Ins(1,3,4,5,6)P_5$-2-kinase (probably does not exist, as $Ins(1,3,4,5,6)P_5$ is unlikely to be the precursor of $InsP_6$); 21, $Ins(3,4,5,6)P_4$-1-kinase, 22, Ins(3)P-synthetase.

the expense of the Ins(1,4,5)P_3-insensitive pool[20] and this process may be controlled by Ins(1,3,4,5)P_4 (ref. 11). The Ins(1,4,5)P_3-insensitive pool may also help to amplify and propagate the calcium signal derived from the Ins(1,4,5)P_3-sensitive store by a mechanism of calcium-induced calcium release (see below).

Ins(1,4,5)P_3-induced calcium mobilization

To release calcium, Ins(1,4,5)P_3 must bind to receptors that are somehow linked to calcium channels connected with the Ins(1,4,5)P_3-sensitive calcium pool. A variety of tissues have a high-affinity stereospecific binding site for Ins(1,4,5)P_3, which recognizes D-Ins(1,4,5)P_3 much better than L-Ins(1,4,5)P_3 (refs 21, 22). This may be the receptor responsible for releasing calcium, because both processes have a similar profile of responses to InsP_3 derivatives and enantiomers[3,21,23]. Heparin also inhibits both the binding of Ins(1,4,5)P_3 to its purified receptor[15] and the mobilization of calcium from liver cells[24].

Individual Ins(1,4,5)P_3-sensitive calcium channels (sensitive to heparin) with conductances of ~10 pico-Siemens (pS) have been recorded from membrane vesicles isolated from the sarcoplasmic reticulum of aortic smooth muscle, which have been incorporated into planar lipid bilayers[25]. The action of Ins(1,4,5)P_3 seems highly cooperative, suggesting that three molecules are required to open the calcium channel[26]. Using a caged molecule from which free Ins(1,4,5)P_3 can be released by a flash of laser light[27], a distinct threshold can be demonstrated above which the response seems to be all-or-nothing[28]; paired light flashes produced facilitation[28]. Overall, this cooperativity may help to explain the long latencies that exist when cells are stimulated with calcium-mobilizing agents[29]. Finally, reconstitution experiments with the purified receptor suggest that the Ins(1,4,5)P_3-binding site, and the Ca^{2+} channel which it controls, may reside in the same protein (C. D. Ferris, R. L. Huganir, S. Supattapone and S. H. Snyder, personal communication).

The Ins(1,4,5)P_3-induced release of calcium can be modulated by a variety of physiological and pharmacological agents. Classical calcium-channel blockers have no effect[30,31], but release is inhibited by cinnarizine and flunarizine[30] and by the potassium channel blocker tetraethylammonium (TEA)[31]. In the absence of potassium, Ins(1,4,5)P_3 is incapable of releasing calcium but other monovalent cations ($Na^+ \gg Tris^+ > Li^+$) can replace potassium, which functions to neutralize the charge developed by the efflux of calcium[32]. In cerebellar membranes, both the binding of Ins(1,4,5)P_3 to its receptor and its calcium-mobilizing action are sensitive to calcium[15,33]. This effect seems to depend on a calcium-mediator protein (calmedin) which is particularly abundant in brain but sparse in peripheral tissues[34]. Also, the Ins(1,4,5)P_3 receptor isolated from brain can be phosphorylated by cyclic AMP-dependent protein kinase, resulting in a decrease in its calcium-mobilizing activity[35].

A characteristic feature of the Ins(1,4,5)P_3 receptor is that it does not desensitize. Any decline in the release of calcium can usually be attributed either to the rapid metabolism of Ins(1,4,5)P_3 (ref. 23) or to the transfer of the mobilized calcium to an Ins(1,4,5)P_3-insensitive pool. A useful analogue for studying calcium mobilization is inositol 1,4,5-trisphosphorothioate which is resistant to metabolism by either the Ins(1,4,5)P_3-5-phosphatase (ref. 36) or the Ins(1,4,5)P_3-3-kinase (ref. 37) and the lack of desensitization of the Ins(1,4,5)P_3 receptor is emphasized by the fact that calcium mobilized by this phosphorothioate derivative is not re-sequestered[37].

Calcium entry—two inositol phosphates involved?

In addition to mobilizing internal calcium through Ins(1,4,5)P_3, many agonists can also promote an influx of external calcium. The three main types of calcium channel used to regulate calcium influx are voltage-operated calcium channels, and two agonist-dependent types which are opened either directly through a receptor (receptor-operated channel) or indirectly through some internal diffusible messenger (second messenger-operated channel)[38]. These calcium channels differ in the way they are activated and, probably, in the very different calcium signals that they generate. Voltage-operated calcium channels, which are usually found in excitable cells, give rapid but brief calcium pulses when activated by membrane depolarization. Opening of receptor-operated channels, however, results in a rapid and maintained elevation in intracellular calcium which is also totally dependent on external calcium. The calcium signal derived from the opening of voltage-operated channels may be amplified by the mobilization of intracellular calcium through a calcium-dependent formation of Ins(1,4,5)P_3 (ref. 39).

There is considerable uncertainty concerning the mechanism and control of second messenger-operated channels. The amount of calcium flowing through these channels is very small, and they do not display the characteristics of typical channels but seem instead to generate a smooth inward current[40]. When cells are stimulated with agonists operating through these channels, the large initial increase in calcium (due mainly to mobilization of internal calcium) is not maintained and the

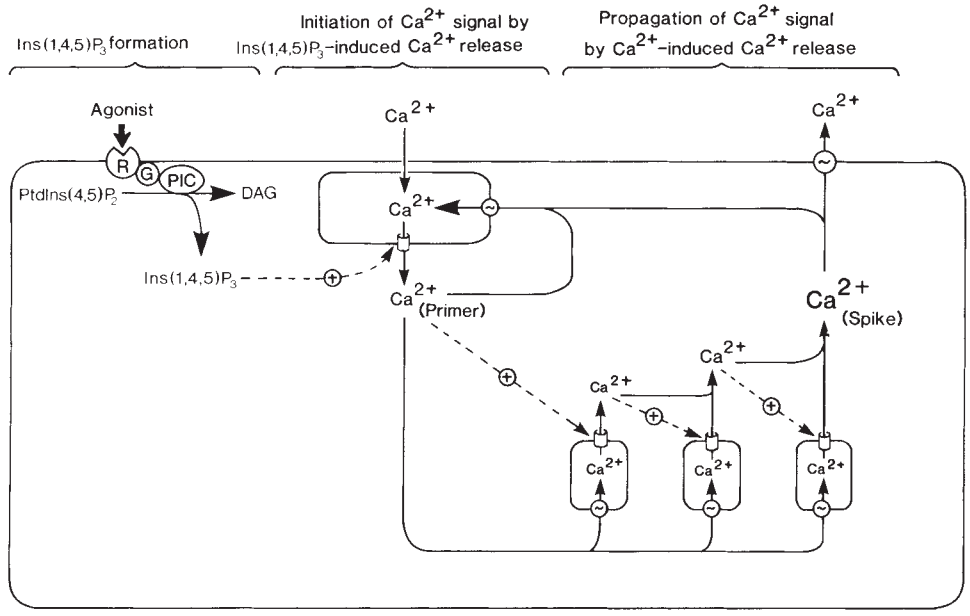


FIG. 2 A unified hypothesis of the spatio-temporal aspects of calcium signalling, based on ideas concerning calcium oscillations[67,79] and the propagation of calcium waves[57–60]. A typical response begins with agonists generating Ins(1,4,5)P_3, which then mobilizes calcium from an Ins(1,4,5)P_3-sensitive calcium store and also promotes the entry of external calcium (see Fig. 3 for details) to give an initial calcium signal. In some cells, this Ins(1,4,5)P_3-induced calcium signal can act as a 'primer' to drive a process of calcium-induced calcium release from the Ins(1,4,5)P_3-insensitive pools to produce a spike which might be organized in the form of a wave, so spreading the signal throughout the cell.

concentration usually declines to a low plateau level. Both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ have been implicated in controlling this slow entry of external calcium. Just how these inositol phosphates may function is confused because the route of calcium entry is unknown. The simplest hypothesis is that calcium flows directly into the cytosol through channels in the plasma membrane (Fig. 3*a*). Such channels could be regulated by $Ins(1,4,5)P_3$ as has been reported in lymphocytes[41] and mast cells[40]. An alternative capacitative mechanism is that calcium flows first into the ER before entering the cytosol[42]. This is an autoregulatory mechanism because the calcium content of ER that is closely apposed to the plasma membrane somehow regulates the flow of calcium into the cell (Fig. 3*b*). Just how calcium traverses both the plasma membrane and the ER membrane is not known but one idea is that it passes through a pore (analogous to a gap junction) linking the two membranes[43]. Consistent with this hypothesis that the emptying of an internal pool is a prerequisite for entry, is the observation that release of calcium from the internal pool of endothelial cells can precede the entry of external calcium[44]; the control of calcium entry would, therefore, be regulated indirectly by the ability of $Ins(1,4,5)P_3$ to discharge the ER of its calcium (Fig. 3*b*).

This proposed role of the ER may help to explain a possible involvement of $Ins(1,3,4,5)P_4$ in regulating calcium entry, because the action of this inositol phosphate seems to require the simultaneous presence of $Ins(1,4,5)P_3$ (refs. 11, 45). $Ins(1,3,4,5)P_4$, at low concentrations and with a high degree of chemical specificity, increases intracellular calcium concentrations provided that a calcium-mobilizing $InsP_3$ is present[45–47]. This is not the result of $Ins(1,3,4,5)P_4$ competing for the phosphatase that it shares with $Ins(1,4,5)P_3$ which would protect the latter compound[47]. At least some, but not all, of the calcium released comes from outside the cell[47] and the current best guess is that $Ins(1,3,4,5)P_4$ controls the transfer of calcium between intracellular pools[11] (Fig. 3*c*). If some of these pools regulate calcium entry through the capacitative mechanism described above, then this may explain the absolute dependency of calcium entry on $Ins(1,3,4,5)P_4$ (ref. 47) (see Fig. 3*c*). The lack of such a dependency on $Ins(1,3,4,5)P_4$ in other experiments[40] may be explained by the slow reversibility of the effects of $Ins(1,3,4,5)P_4$ once they are initiated[48]. Even if $Ins(1,3,4,5)P_4$ is essential for inositol phosphate-controlled calcium entry, its contribution to intracellular calcium mobilization is, as yet, unknown. Finally, we should note that the idea of $Ins(1,3,4,5)P_4$ linking pools, may also provide an explanation for the increased sequestration of calcium caused by this inositol phosphate in a permeabilized cell preparation[49].

Clearly, there is still a lot to learn about how second messengers regulate calcium entry to maintain the signalling system during prolonged periods of stimulation. The relatively small influx of calcium through second messenger-operated channels does not normally result in marked elevation of intracellular calcium, but it does drive calcium oscillations in many cells by providing a steady input of calcium which is then periodically released to give large but transient calcium spikes (see below).

TABLE 1 Calcium wave propagation velocities

Cell type	Temperature (°C)	Propagation velocity ($\mu m\ s^{-1}$)	Reference number
Rat cardiac myocytes	23 30	33 74	60
Rat cardiac trabeculae	21	50–1,500	58
Hamster egg	30–32	16–28	52
Medaka egg	26	12	66
Xenopus egg	22.5	8–10	57
Sea urchin egg	16.0	5	65
Tracheal epithelium	37	10	85
Endothelial cell	37	50	62
Gonadotropes	22	45–207	*

* D. Leong and M. Thorner, personal communication.

Spatial organization and calcium waves

With the advent of calcium-sensitive indicators and image analysis it has become possible to study how calcium signals are distributed in space and time within a single cell[50]. Calcium signals may either be uniform, or they can initiate in discrete regions from which they may propagate in the form of waves. In bovine adrenal cells, for example, the opening of voltage-operated channels gives a uniform calcium signal, whereas the activation of muscarinic receptors results in a highly localized rise in calcium[51] (Fig. 4). Such discrete initiation sites have been observed in other cells responding to stimuli which hydrolyse inositol lipids, for example, in eggs[52], adrenal glomerulosa cells[53], *Limulus* photoreceptors[54] and NIH-3T3 cells[55]. This spatial organization of calcium signals may be determined by an unequal distribution either of the receptor mechanisms responsible for generating $Ins(1,4,5)P_3$ or of the $Ins(1,4,5)P_3$-sensitive calcium pools[56]. Fertilization is an example where the external signal dictates the initiation site of the calcium signal; this begins at the point of sperm–egg fusion from where it spreads as a propagated wave towards the opposite pole[52,57]. Calcium waves also occur in cardiac muscle[58], myocytes[59,60], gonadotropes[61], endothelial cells[62] and hepatocytes[63]. In all these cells, the waves propagate in a non-decremental way at very similar velocities (Table 1), suggesting a common mechanism, which could be a well-ordered system of calcium pools aligned to allow calcium to spread from one pool to another (Fig. 2). In myocytes, the sarcomeric organization of the myofibrils separates the sarcoplasmic reticulum into regular arrays. *Xenopus* eggs also have a regularly spaced arrangement of the ER lined up close to the plasma membrane[64]. The higher density of ER in the animal as compared with the vegetal hemisphere, may explain how the wave propagates faster in the former[64].

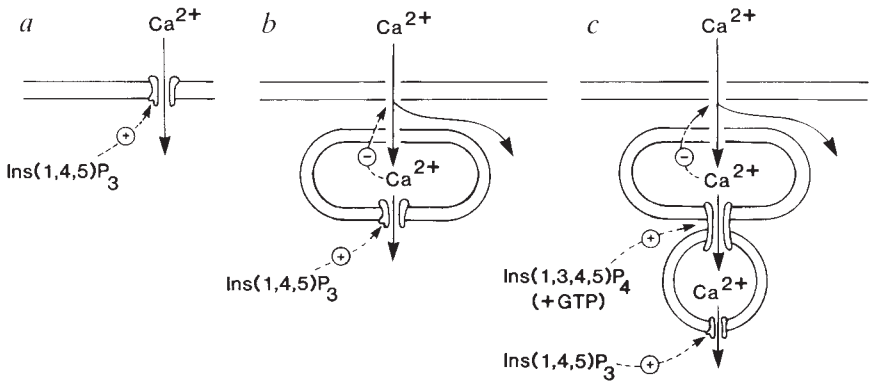


FIG. 3 Summary of the proposed mechanisms by which inositol phosphates control calcium entry into cells. *a*, $Ins(1,4,5)P_3$ controls a calcium channel in the plasma membrane[40,41]; *b*, a capacitative model, in which $Ins(1,4,5)P_3$ controls a channel in an endomembraneous compartment which indirectly regulates calcium entry across the plasma membrane through the operation of a negative-feedback loop[42]; *c*, extension of Putney's capacitative model to include a role for $Ins(1,3,4,5)P_4$ in controlling the transfer of calcium between pools[11,45].

There are two principal models of how calcium waves might spread through cells. One suggestion is that $Ins(1,4,5)P_3$ and calcium act as positive regulators of each other[65]. An essential feature of this model is that calcium activates the production of $Ins(1,4,5)P_3$. Although an increase in calcium is not obligatory for the activation of phosphoinositidase C, elevating calcium can enhance the formation of $Ins(1,4,5)P_3$ in certain cells[39]. The other model, to us more attractive, is that the wave spreads by a process of calcium-induced calcium release coupled by diffusion as has been suggested for eggs[52,57,66] and for contractile cells[58,59]. Calcium elevated at a discrete initiation site diffuses to an adjacent store to stimulate the release of calcium and so on down the array[58,59] (Fig. 2). Under circumstances where there are multiple initiation sites, separate waves may result, and these can collide and annihilate each other[59]. This indicates that there is a refractory period following the wave[59], which probably depends on the time required to recharge the calcium stores to the point at which they can once again respond to a calcium signal. We argue below that $Ins(1,4,5)P_3$ may play a key role in priming these stores.

Calcium oscillations

The intracellular level of calcium often oscillates, especially when the phosphoinositide pathway is being stimulated[29,67]. There are many oscillatory patterns, but usually they have the form of a constant baseline which is periodically interrupted by calcium spikes. In contrast to the more classical membrane oscillators which operate through a phasic opening of voltage-operated calcium channels in the plasma membrane, the oscillatory mechanism we are considering here is based on the periodic release of internal calcium (cytosolic oscillators). Most oscillations have a period of between 5 and 60 s (ref. 29) but it is particularly noteworthy that this frequency depends on agonist concentration in some tissues[68–71] but not others[72,73]. Whereas the form and frequency of the oscillations vary from cell to cell, they are remarkably constant for individual cells responding to repeated application of a specific agonist, and have, therefore, been referred to as 'fingerprints'[71]. Some oscillations cease almost immediately when external calcium is removed[70] whereas others persist for a considerable time[67]. All these differences suggest that oscillations may be generated by different mechanisms under different circumstances[29]. In most of the models developed so far, $Ins(1,4,5)P_3$ is a key regulator of the periodic release of internal calcium; however, there is considerable controversy over whether the concentration of $Ins(1,4,5)P_3$ oscillates[67].

In receptor-controlled oscillator models, feedback loops that control the hydrolysis of $PtdIns(4,5)P_2$ by phosphoinositidase C are invoked, that cause the concentration of $Ins(1,4,5)P_3$ to oscillate. This may be through a negative feedback loop using the DAG/protein kinase C pathway[29,74], or calcium may be a positive regulator of $Ins(1,4,5)P_3$ formation[75]. In the latter hypothesis, an important consideration is the strong cooperative interaction between $Ins(1,4,5)P_3$ and the receptor through which it mobilizes calcium[26]. A third model[45,73] invokes the calcium-dependent conversion of $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ (ref. 76), which may act as a feed-forward signal to promote further calcium mobilization (see above). An attractive feature of this model is that the calcium spike will not be maintained because full activation of the $Ins(1,4,5)P_3$-3-kinase would soon exhaust all the $Ins(1,4,5)P_3$ and terminate further release because $Ins(1,3,4,5)P_4$ cannot mobilize calcium on its own. However, the surge in $Ins(1,3,4,5)P_4$ may set the stage for the next transient by helping to load the $Ins(1,4,5)P_3$-sensitive pool both by stimulating the re-uptake of calcium into this pool[49] and by promoting the transfer of calcium between intracellular pools[11,45].

Present techniques are not sensitive enough to determine whether $Ins(1,4,5)P_3$ is oscillating in single cells. As oscillations can be observed when a variety of cells are injected or perfused with $Ins(1,4,5)P_3$, receptor-controlled models seem unlikely, at least in these systems[67,77]. In particular, in pancreatic cells, identical oscillations to those produced by acetylcholine were induced by perfusing the cell with the non-metabolizable phosphorothioate derivative of $Ins(1,4,5)P_3$, thus making less likely a role for fluctuations in $Ins(1,4,5)P_3$ or $Ins(1,3,4,5)P_4$ (ref. 77).

Second-messenger controlled models propose that the oscillator resides within the ER/SR system which releases calcium periodically in response to a steady level of $Ins(1,4,5)P_3$. One proposal[33,71,72,78] is that release may become periodic because a negative calcium feedback is known to inhibit both the binding and the calcium-mobilizing action of $Ins(1,4,5)P_3$ (refs 15, 33). Another model, based on that discussed above for the initiation and propagation of a calcium wave in *Xenopus* eggs[57], considers that oscillations arise through an interplay between different calcium pools (Fig. 2). As the $Ins(1,4,5)P_3$-sensitive pool empties it primes the $Ins(1,4,5)P_3$-insensitive calcium pool which then begins to release calcium in an oscillatory manner through the

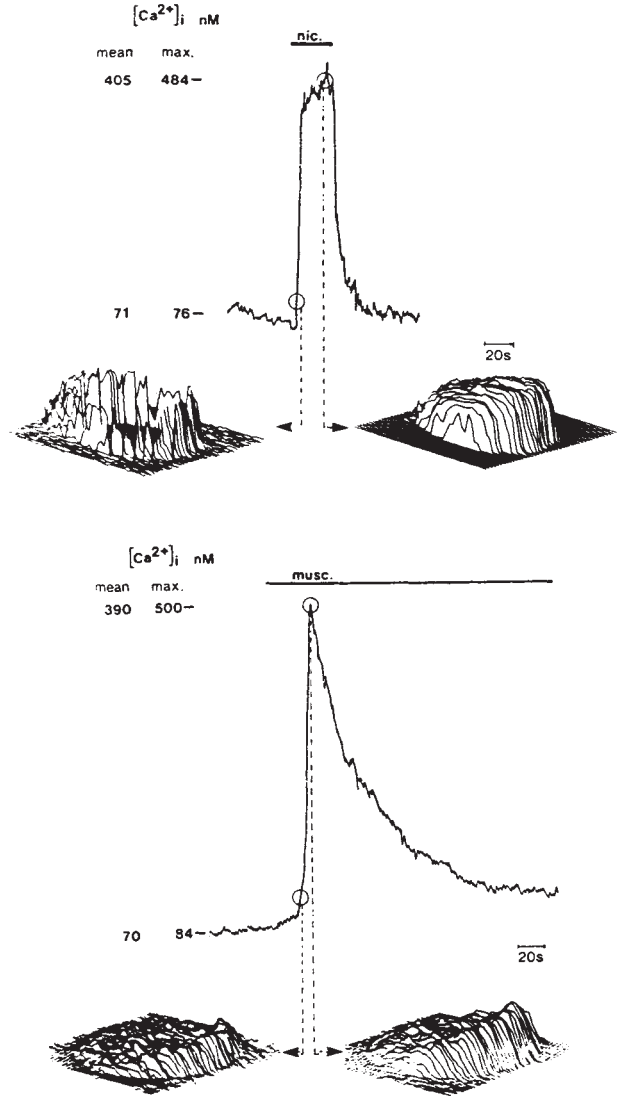


FIG. 4 Spatial organization of calcium signalling in a bovine adrenal chromaffin cell during stimulation with either nicotine (nic) or muscarine (musc). Although these two stimuli gave comparable mean and maximal calcium signals, the contour maps reveal that cellular distributions of calcium during the onset (left) and at the peak of the response (right) are very different. During nicotine stimulation, which results in the opening of voltage-operated channels, calcium rises initially around the periphery and then the interior fills in to give a uniform elevation throughout the cell. On the other hand, the increase in calcium following muscarinic stimulation is highly localized, in the form of a crescent, to one region of the cell. (Unpublished, using data taken from ref. 51).

process of calcium-induced calcium release[29,67] which has previously been proposed for setting up oscillations in various cell types[56,59,79-81].

Just how calcium release is triggered is uncertain. Whatever the activation mechanism is, it must be related in some way to the filling of the internal store to some constant level to account for the observation that both the rate of rise and the amplitude of the individual calcium spikes remain constant despite the large changes in frequency induced by varying agonist concentrations[69,70,79]. The build-up of calcium in the ER compartment somehow precipitates a sudden release of calcium either because the concentration within the cisternae reaches some critical threshold or because the saturation of the uptake system nullifies its buffering capacity so that cytosolic calcium is elevated, and this provides the activation signal (Fig. 2). The existence of a 'pacemaker' calcium ramp preceding the onset of the calcium spike in endothelial cells supports this latter mechanism[70].

Models based on calcium-induced release are attractive because this process can account for many of the characteristics of calcium oscillations and also for how these can be induced by stimuli other than those which act through inositol lipid hydrolysis. Because oscillations are apparently a calcium-overload response, repetitive calcium spiking will follow any stimulus which increases intracellular calcium, such as potassium depolarization in sympathetic ganglia[80] and smooth muscle[82], or by placing mammalian cardiac muscle in low sodium media or treating it with ouabain[59]. The period between spikes will be determined by how long it takes to charge up (prime) the $Ins(1,4,5)P_3$-insensitive pool. The external concentration of calcium will influence oscillator frequency depending on the extent to which it contributes to the loading of this pool[67]. If the oscillator is reasonably self-contained, that is, most of the calcium in the spike is returned to internal pools, removing external calcium will have little immediate effect. If, on the other hand, most of the calcium is extruded from the cell following each transient, oscillator frequency will be very dependent on external calcium, as it is in myocytes[59], endothelial cells[70] and hepatocytes[79]. The remarkable agonist-specific shapes of individual calcium transients in hepatocytes[74,79] may be explained by agonists having variable effects on the rate of calcium extrusion from cells[79].

Calcium oscillations can be considered as a transformation of the calcium signal into a digital form whose frequency varies with agonist concentration. Calcium signalling may therefore be frequency-coded and the advantages of using frequency instead of amplitude are discussed in detail elsewhere[29,68-70]. Such oscillatory activity may not only mediate the action of neurotransmitters and hormones, but may also be important in regulating cell growth. Prolonged oscillations have been described in lymphocytes responding to antigen[83] and also during interphase of Swiss-3T3 cells, particularly as the cells exit from mitosis[84].

A unifying hypothesis

The distribution of calcium signals in space and time seem to be reflections of a common mechanism, and so we can begin to formulate a unified hypothesis[59,67,79]. Each transient during a calcium oscillation can be spatially organized, as it is often initiated at a discrete site from which it then spreads as a wave throughout the cell. This sequence of events begins with the agonist-stimulated formation of $Ins(1,4,5)P_3$ which activates the process of calcium-induced calcium release that is responsible for the spatially-organized calcium transient as discussed above (Fig. 2). Although the significance of the different components may vary from cell to cell, there is every reason to suppose that both oscillations and signal propagation through calcium waves share a common mechanism.

So far we have concentrated on events in a single cell, but many cells are connected together by means of gap junctions which may allow cells to function in unison with regard to both oscillatory activity and wave propagation. Evidence that a calcium wave may travel from one cell to the next has come from studies of the ciliated tracheal epithelium[85]. Mechanical excitation of one cell accelerates its ciliary beat through an increase in intracellular calcium. A wave of excitation then spreads to neighbouring cells at a rate of ~ 10–$15\ \mu m\ s^{-1}$ (ref. 85). This rate is similar to that for the intracellular waves described earlier (Table 1), so it seems that propagation may spread from one cell to the next, provided that sufficient components of the wave ($Ins(1,4,5)P_3$ or calcium) can flow through the gap junctions. Gap junctions are known to provide an avenue for intercellular stimulation by cyclic AMP (ref. 86) and there is evidence that both $Ins(1,4,5)P_3$ and calcium can flow down a chain of hepatocytes, sometimes in the form of a wave[63]. Intercellular communication of $Ins(1,4,5)P_3$ or calcium may also explain the synchronized calcium oscillations described in endothelial monolayers[87] and the oscillatory release of calcium from the intact liver following hormonal stimulation[88]. It is unclear whether such synchronization arises because all the cells are oscillating in phase, or whether there is a 'pacemaker' centre which entrains neighbouring oscillators by passing information along gap junctions, but either way, the spatiotemporal hypothesis of calcium signalling may help to explain the way in which cell populations communicate with each other to produce synchronous responses.

Why so many inositol phosphates?

Cells contain a bewildering array of inositol phosphates[10-12] which seem to fall into two functional groups (Fig. 1): those whose levels change in response to agonist stimulation and thus have functions related to intracellular signalling, and the agonist-insensitive group concerned with the synthesis of $InsP_5$ and $InsP_6$ whose levels, if they change, do so comparatively slowly during stimulation. In the agonist-sensitive group, the first complication is that, in addition to $Ins(1,4,5)P_3$ being formed by phosphoinositidase C activity on $PtdIns(4,5)P_2$, some cyclic inositol trisphosphate ($Ins(1:2cyc,4,5)P_3$) is also released[89]. $Ins(1:2cyc,4,5)P_3$ is formed very slowly[90,91], but as it is a poor substrate for the phosphatase and kinase that metabolize $Ins(1,4,5)P_3$ (refs 11, 12), it can eventually accumulate to reach a concentration similar to that of $Ins(1,4,5)P_3$, after prolonged stimulation[90]. Although $Ins(1:2cyc,4,5)P_3$ can mobilize calcium[89], its potency is probably at least an order of magnitude less than $Ins(1,4,5)P_3$ (ref. 92). The available evidence, therefore, indicates that cells cannot avoid making cyclic inositol phosphates (because of the way phosphoinositidase C works) but that they are unlikely to use them for any physiological purpose.

Much of the remaining proliferation of agonist-sensitive inositol phosphates originates from the metabolism of $Ins(1,4,5)P_3$ being through two separate pathways (Fig. 1). It can be sequentially dephosphorylated to free inositol (enzymes 10 and 11, Fig. 1) or it can be phosphorylated to $Ins(1,3,4,5)P_4$ by an $Ins(1,4,5)P_3$-3-kinase (enzyme 15, Fig. 1). The latter is strongly activated in v-*src* transformed cells resulting in a sevenfold elevation in the level of an $InsP_4$ (ref. 93). The hydrolysis of $Ins(1,3,4,5)P_4$ to $Ins(1,3,4)P_3$ is a straightforward reaction, but much of the complexity arises because the latter can be hydrolysed back to inositol by separate routes (Fig. 1). What is the significance of this complexity? At this stage we can offer two extreme viewpoints. The complexity could arise because one or more of the breakdown products themselves have physiological functions. One could argue teleologically that this would be undesirable, because the only route of synthesis of any of these other inositol phosphates is through a known second messenger, $Ins(1,4,5)P_3$, so an increase in another putative second messenger would always have to be associated with a rise in calcium. We think that it is more likely that the complexities are simply an inevitable result of the 3-phosphorylation of $Ins(1,4,5)P_3$ to form $Ins(1,3,4,5)P_4$: if the cells are coping

with the formation of two second messengers—Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4—rather than one, more enzymes will be required to catabolize these, and so a complex pattern of intermediates cannot be avoided[11].

$InsP_5$ and $InsP_6$

The metabolism of $InsP_5$ and $InsP_6$ occurs through pathways which are largely separate from the agonist-sensitive pathways (Fig. 1). The main route by which these compounds are made in animal cells is not yet clear and may involve separate pathways for $InsP_5$ and $InsP_6$. In the case of $InsP_5$, a pathway exists from agonist-sensitive inositol phosphates through the phosphorylation of Ins(1,3,4)P_3 to Ins(1,3,4,6)P_4 and thence to Ins(1,3,4,5,6)P_5 (ref. 96) (Fig. 1). However, the main route of synthesis of this inositol phosphate is probably through Ins(3,4,5,6)P_4, which has long been known to be a constituent in avian erythrocytes along with $InsP_5$ (ref. 95) and which also exists in mammalian cells, together with an active and specific kinase which converts it to $InsP_5$ (ref. 94). WRK-1 cells have very high levels of Ins(3,4,5,6)P_4 which increase slowly following stimulation with vasopressin[97]. But the origin of Ins(3,4,5,6)P_4 is, as yet, unknown; that is, we do not know if it is formed from phosphorylation of inositol or indirectly from Ins(1,4,5)P_3. $InsP_6$ is very likely to be generated by phosphorylation of inositol. $InsP_5$ and $InsP_6$ incorporate radiolabel more slowly than 'receptor-generated' inositol phosphates, but they can eventually incorporate [^{3}H]inositol up to a very high level[98], consistent with their high (millimolar) mass levels in the cell[99] (compare for example the micromolar levels of Ins(1,4,5)P_3 (ref. 100)). The proportion of $InsP_5$ to $InsP_6$ changes markedly when HL60 cells begin to differentiate[101]. The overall picture we have of these compounds is, therefore, one of a high resting level which, at least for Ins(1,3,4,5,6)P_5, can perhaps be changed after prolonged stimulation by synthesis from Ins(1,3,4)P_3.

$InsP_5$ and $InsP_6$ are therefore generally assumed to have a 'housekeeping' function rather than being acute second messengers. Speculations about their function have abounded, but perhaps the most promising for $InsP_6$ centres around its unique anti-oxidant properties[102]. Recently it has been suggested that $InsP_5$ and $InsP_6$ are extracellular signals, neurotransmitters[103] for example, but even if correct, this exciting discovery does not help to clarify their intracellular roles.

Lipid diversity and enzyme heterogeneity

Phosphatidylinositol has a rich diversity of forms and functions within the cell, as do the enzymes associated with its metabolism. Apart from its structural role as part of the lipid bilayer, PtdIns is a precursor of second messengers and is a membrane anchor for a great variety of cell surface proteins[104]. This functional diversity is associated with structural variations of both the inositol head group and the fatty-acid tail regions. For example, the PtdIns used for protein anchoring has a fatty-acid composition rich in myristic acid, whereas that attached to PtdIns(4,5)P_2 is predominantly stearoyl–arachidonyl in composition.

A major function of inositol lipids is the one principally under discussion here, to supply second messengers such as Ins(1,4,5)P_3 and DAG in response to calcium-mobilizing agonists[1–7]. For this function, PtdIns is phosphorylated to PtdIns(4)P and then to PtdIns(4,5)P_2; the latter is the precursor used for signal transduction (Figs 1 and 2). The recent discovery of a new lipid, PtdIns(3)P, has emerged from work being carried out on the function of the proto-oncogene c-*src* (ref. 105). The product of c-*src* in association with the middle T antigen of SV40, acts to phosphorylate (and thus possibly to activate) type I PtdIns kinase, which is distinct from the type II enzyme responsible for forming PtdIns(4)P, not least because it phosphorylates PtdIns on the 3-position to give PtdIns(3)P (Fig. 1). A possible role for type I kinase in cell proliferation[105] has recently received strong support from studies which show that platelet-derived growth factor (PDGF) receptors that are deficient in their ability to associate with this kinase fail to stimulate growth[106]. There is also a report of the transient appearance of a PtdIns containing 4 phosphates (possibly PtdIns(3,4,5)P_3) in stimulated neutrophils[107] and of a possible PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in PDGF-stimulated fibroblasts[108]. It is tempting to speculate whether such novel lipids fulfil some of the other proposed functions of PtdIns(4,5)P_2, such as remodelling the cytoskeleton[109,110], without compromising the established function of PtdIns(4,5)P_2 as a precursor for DAG and the inositol polyphosphates.

The protein-anchoring function of PtdIns glycans serves as a mechanism for quickly converting a protein which does not have a predominantly hydrophobic region, into a membrane-bound form. It also provides a means for the rapid release of proteins from membranes by reactions catalysed by phospholipase C, as is observed during the change of the protein coat in trypanosomes[104]. Also, the insulin-sensitive hydrolysis of a glycosylated PtdIns releases an inositol phosphate glycan which might be a second messenger for some of the actions of insulin[104].

The growing diversity of the inositol lipids is more than matched by the number of enzymes now known to be responsible for both the initial production and subsequent metabolism of Ins(1,4,5)P_3 (Fig. 1). This complexity is increased because there are different molecular forms of many of these enzymes, for example Ins(1,4,5)P_3-5-phosphatase[111] (enzymes 10 and 11, Fig. 1) and PtdIns synthetase[112] (enzyme 1, Fig. 1). The recent discovery of a possible function of inositol lipids in the nucleus[113] may itself spawn an entirely new family of enzymes. Indeed, Sylvia *et al.*[114] have reported that Ins(1,4)P_2 or its parent lipid PtdIns(4)P can specifically activate a DNA polymerase.

The molecular basis for this enzyme heterogeneity is being studied by genetic techniques which have already revealed that protein kinase C is a heterogeneous enzyme family, with a distinct, and in some cases very precise, tissue specificity[2]. Phosphoinositidase C also exists in a remarkable number of different forms[115] and at least one of these may have coding regions resembling the tyrosine kinase-related oncogenes. Studies on the *Drosophila NorpA* mutant, which is blind but otherwise normal, illustrate the degree to which this phosphoinositidase C family may be tissue-specific. When Yoshioka and colleagues showed that these flies lack phosphoinositidase C in their eyes and suggested that *NorpA* might be a structural gene for this enzyme[116], the idea was received with some sceptism because it would require a phosphoinositidase C gene specific only for photoreceptors. The recent genetic confirmation that *NorpA* does code for a protein with very close similarities to known phosphoinositidase C enzymes, and the observation that transcripts of the gene were localized to the eye and a small region of the brain, suggests that such tissue specificity might indeed exist[117].

Lithium

When administered to intact organisms, lithium induces subtle alterations in neural activity (for example, in manic-depressive illness and diurnal rhythms) and early development (teratogenesis). Lithium is known to reduce the supply of inositol, the key substrate for the phosphoinositide cascade, by inhibiting some of the enzymes (enzymes 13 and 14, Fig. 1), which hydrolyse the inositol phosphates. The 'inositol depletion hypothesis' may explain the neural and developmental effects of lithium on the basis that it inhibits signal transduction indirectly by slowing down the supply of the precursor lipid required to generate messengers such as Ins(1,4,5)P_3 and DAG (ref. 118). The subtlety of the action of lithium is that it seems to be selective; it ignores those receptors which are operating normally, but 'searches out' and dampens down those which are overactive. This selectivity is enhanced by the inhibitory action of lithium being uncompetitive[119] with the result that its effect is progressively enhanced as the level of substrate increases. It is this selective action against overactive receptors

which is the key feature of the inositol depletion hypothesis. Those hypotheses which attempt to explain how lithium might act through a direct effect on signal-transducing elements (adenylyl cyclase or G proteins) cannot satisfactorily explain why lithium has no acute effect on a variety of cellular responses, nor why it has little or no effect on people not suffering from manic-depressive illness. However, it is not presently clear how the apparent tissue (brain)- and agonist (carbachol)-specific inhibition by lithium of inositol phosphate formation[120], is related to its effects on inositol phosphate phosphatases, nor to the influence of lithium on manic depression.

The teratogenic effect of lithium has been investigated in *Xenopus* where its effect is to respecify the dorsal-ventral axis which is fixed at the time of fertilization. This polarity in the early embryo is based on some positional variable or 'organizer' set up in the form of a gradient along the dorsal-ventral axis. More specifically, Busa[121] has proposed that phosphoinositide-derived messengers such as Ins(1,4,5)P_3 or DAG might be the morphogens responsible for early pattern formation. On the basis of the inositol depletion hypothesis, the action of lithium can be interpreted by supposing that it flattens out a normal gradient of phosphoinositide turnover which is postulated to be high in the ventral pole and low in the dorsal pole. Conversely, UV-irradiation which promotes ventralization may produce a uniformly high level of phosphoinositide turnover.

A gradient of phosphoinositide turnover along the dorsal-ventral axis is entirely consistent with the distribution of junctional permeability in the early embryo. Activation of the phosphoinositide messenger pathway, particularly the DAG protein kinase C branch, is known to decrease the permeability of gap junctions[122]. It is significant, therefore, that transfer of the dye Lucifer Yellow between cells in the ventral region is less than in the dorsal region[123]. If pattern formation depends upon messengers such as Ins(1,4,5)P_3 and calcium, which can diffuse between cells (see above), then this reduction in junctional permeability in the ventral pole may serve to confine these morphogens to this region. Treatment with lithium gives a uniformly high level of dye transfer, whereas UV-irradiation reduces transfer[123] and this is exactly in line with the proposal that these two treatments even out the gradient of phosphoinositide turnover to levels which are either uniformly low (lithium) or high (UV-irradiation).

The hypothesis based on a gradient of phosphoinositide turnover is also consistent with experiments where lithium has been injected into one or other pole[121,124,125]. Dorsalization induced by injecting lithium specifically into the ventral pole can be prevented if the lithium is co-injected with *myo*-inositol (but not *epi*-inositol)[125], an observation that provides strong support for the inositol depletion hypothesis. Furthermore, it suggests that a gradient of phosphoinositide messengers may be pivotal in pattern formation during embryogenesis.

Conclusion

In reviewing our current understanding of the role of Ins(1,4,5)P_3 in regulating physiological functions of cells *in vivo*, we have highlighted many complexities: multiple calcium pools, the interrelationship between calcium mobilization and entry, and several possible mechanisms of generating calcium oscillations and waves. Pervading all these aspects to an, as yet unknown degree is the probable helper function of Ins(1,3,4,5)P_4. These subtleties and complexities suggest a reason why the inositol phosphate signalling system is so widespread in biology. It gives to cells the ability to do so much more than just to raise or lower calcium levels. This in turn provides the means to generate an enormous variety of cellular responses from one essentially straightforward set of signals. For example, a cell may distinguish one receptor from another, even though both are working through inositol phosphates. It may use the same signals to control short-term functions (such as secretion or metabolism) and long-term functions (such as growth), and the interactions between different intracellular signals could be virtually limitless in their variety.

The complicated biochemistry of inositol phosphates illustrates the extraordinary diversity of compounds into which *myo*-inositol can be incorporated. Here we have limited our survey mostly to vertebrates, but many more inositol-containing compounds exist in other organisms, particularly in plants. We know very little about how these many compounds are formed, and we are just beginning to fathom functions for only a few of them. In vertebrates, a main challenge for the future is to reveal the wider implications of the phosphoinositide signalling system in the control of complex biological processes, such as early development and the neural mechanisms which underly behaviour. □

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ARTICLES

A 42K outer-membrane protein is a component of the yeast mitochondrial protein import site

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An engineered precursor protein that sticks in the import site of isolated yeast mitochondria can be specifically photo-crosslinked to a mitochondrial outer-membrane protein of relative molecular mass 42,000 (42K). This protein (termed import-site protein 42 or ISP 42) is exposed on the mitochondrial surface; antibodies against it block protein import into mitochondria. ISP 42 is the first identified component of the putative transmembrane machinery that imports proteins into mitochondria.

MITOCHONDRIA take up almost all of their proteins from the cytosol[1]. One of the earliest steps in this process is the interaction of protein precursors with receptor-like proteins on the mitochondrial surface[2,3]; none of these proteins has so far been unambiguously identified, although it has been demonstrated that protein import into isolated yeast mitochondria is inhibited by antibodies or Fab fragments raised against a group of outer membrane proteins[4] of relative molecular mass (M_r) 45K. It has also been reported that a 30K protein of mammalian mitochondria[5] or of chloroplasts[6] interacts with a chemically synthesized targeting peptide specific for the corresponding organelle. There is no direct evidence, however, that any of these polypeptides are components of the protein import machinery.

We report here that a 42K protein (ISP 42) of the yeast mitochondrial outer membrane can be specifically crosslinked to a precursor protein which is stuck in the import sites and that antibodies against ISP inhibit import of precursors into mitochondria. ISP 42 is thus a component of the mitochondrial protein-import system.

The translocation intermediate

The chimaeric precursor[7], used to investigate the import site, consisted of a fusion protein[8] with the C terminus crosslinked to bovine pancreatic trypsin inhibitor (BPTI). The fusion protein contained the presequence of yeast cytochrome oxidase subunit IV (coxIV a mitochondrial inner-membrane protein) —joined to the N terminus of mouse dihydrofolate reductase (DHFR a

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