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Inositol Phosphates: Proliferation, Metabolism and Function [and Discussion]

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Inositol phosphates: proliferation, metabolism and function

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After the initial discovery of receptor-linked generation of inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3) it was generally assumed that Ins(1,4,5)P_3 and its proposed breakdown products inositol(1,4)bisphosphate (Ins(1,4)P_2) and Ins1P, along with cyclic inositol monophosphate, were the only inositol phosphates found in significant amounts in animal cells. Since then, three levels of complexity have been introduced. Firstly, Ins(1,4,5)P_3 can be phosphorylated to Ins(1,3,4,5)P_4, and the subsequent metabolism of these two compounds has been found to be intricate and probably different between various tissues. The functions of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are almost certainly to regulate cytosolic Ca^{2+} concentrations, but the reasons for the labyrinth of the metabolic pathways after their deactivation by a specific 5-phosphatase remain obscure. Secondly, inositol pentakis- and hexakisphosphates have been found in many animal cells other than avian erythrocytes. It has been shown that their synthesis pathway is entirely separate from the inositol phosphates discussed above, both in terms of many of the isomers involved and probably in the subcellular localization; some possible functions of InsP_5 and InsP_6 are discussed here. Thirdly, cyclic inositol polyphosphates have been reported in stimulated tissues; the evidence for their occurrence *in vivo* and their possible physiological significance are also discussed.

1. Introduction

Six years ago, at a meeting similar to this one, Michell *et al.* (1981) suggested that when an agonist-occupied receptor activated an inositol-lipid phosphodiesterase, the primary substrate was not PtdIns, but was rather PtdIns(4,5)P_2, with the consequent formation of diaclyglycerol and Ins(1,4,5)P_3. Since then, the suspected function of Ins(1,4,5)P_3 has emerged (Berridge 1983; Streb *et al.* 1983) and is now generally accepted (see Berridge & Irvine 1984; Berridge 1987; Irvine 1988). However, in the discussion in Michell *et al.* (1981) the metabolic scheme of inositol phosphates included only Ins(1,4,5)P_3, Ins(1,4)P_2 and Ins1P. Together with cycIns(1–2)P (Dawson *et al.* 1973), these were generally assumed to be the only inositol phosphates in mammals.

Recently, the number of inositol phosphates known to exist naturally has proliferated and it now stands at over 20; subsequent studies have shown these to be connected by a complex metabolic network. The involvement of other inositol phosphates was indicated by several key observations: (1) the discovery of a second inositol trisphosphate, Ins(1,3,4)P_3 (Irvine *et al.* 1984); (2) the discovery that InsP_5 and InsP_6 were not confined to plants and avian erythrocytes (Heslop *et al.* 1985; Morgan *et al.* 1987); and (3) the demonstration that cyclic inositol polyphosphates can be generated in addition to cycIns(1–2)P (Wilson *et al.* 1985*a*, *b*).

These observations, with the more detailed examination of Ins(1,4,5)P_3 metabolism, have led

to the current complicated scheme. Here we attempt, firstly, to review our current understanding of inositol phosphate metabolism, and secondly, through interpretation of experimental data and with some speculation, to suggest possible functions of other inositol phosphates.

2. Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4: a duet in calcium regulation?

After the discovery of Ins(1,3,4,5)P_4 (Batty *et al.* 1985) the possible function of this inositol phosphate has been a source of considerable speculation. Its rapid metabolism in stimulated tissues (Batty *et al.* 1985; Hawkins *et al.* 1986; Hansen *et al.* 1986; Stewart *et al.* 1986) makes it a more likely candidate than its breakdown product Ins(1,3,4)P_3 for being a second messenger; the metabolism of this latter compound is slow and its presence in tissues varies from undetectable, to concentrations as high as 50 μM (compare, for example, Burgess *et al.* 1984, 1985; Irvine *et al.* 1985; Heslop *et al.* 1985; and see Tarver *et al.* 1987). The structure of Ins(1,3,4,5)P_4 suggested that its route of synthesis might be a 3-phosphorylation of the inositol ring of either the parent lipid (PtdIns(4,5)P_2) or the free Ins(1,4,5)P_3 itself. The demonstration of Ins(1,4,5)P_3-3-kinase activity soon followed (Irvine *et al.* 1986; Hawkins *et al.* 1986; Hansen *et al.* 1986; Stewart *et al.* 1986; Biden & Wollheim 1986), and because Ins(1,3,4,5)P_4 was derived directly from Ins(1,4,5)P_3, the physiological significance of this reaction was considered to be closely related to the function of Ins(1,4,5)P_3.

The initial demonstration of a specific biological effect of Ins(1,3,4,5)P_4 was in its ability to activate eggs of the sea urchin *Lytechinus variegatus*, activation being defined as the raising of a visible fertilization envelope; Ins(1,3,4,5)P_4 was only effective if Ins(2,4,5)P_3 was co-injected and if Ca^{2+} was present in the seawater in which the eggs were bathed (Irvine & Moor 1986). It was further shown that the requirement for Ins(2,4,5)P_3 (a poor substrate for InsP_3-3-kinase (Irvine & Moor 1986)) was probably not because Ins(1,3,4,5)P_4 needed increased $[Ca^{2+}]_i$ in the cell, but rather that, for some specific reason, the presence of an inositol trisphosphate was necessary; this led us to propose a coupled mechanism for receptor-stimulated Ca^{2+} control, in which the Ins(1,4,5)P_3-sensitive Ca^{2+} pool is reloaded by an Ins(1,3,4,5)P_4-controlled process (Irvine & Moor 1987; Irvine 1987).

We should note at this point that the synergism of the two inositol phosphates is not a universal feature of all sea urchin eggs. We and others have subsequently found (Irvine & Moor 1987; Crossley *et al.* 1988) that eggs of *Lytechinus pictus* or *Psammechinus milaris*, or even of *Lytechinus variegatus* in the months from February to April, can be activated entirely through mobilization of intracellular Ca^{2+} pools by microinjection of Ins(2,4,5)P_3 on its own, with no evidence for a synergism with Ins(1,3,4,5)P_4 (compare Irvine & Moor 1986). Seasonal effects are well known in sea urchin eggs, and our original experiments (Irvine & Moor 1986, 1987) were done with eggs obtained from animals shipped from Miami in August to October inclusive, at a time at which the animals have shed all of their mature eggs and are producing new ones. The uncovering of a biological effect of Ins(1,3,4,5)P_4 was fortuitous, considering the differences that exist in the physiology of eggs from sea urchins. However, their variability makes these eggs unsuitable as a model system for a comprehensive study of the role of Ins(1,3,4,5)P_4, and other systems must be developed.

To this end, a synergism between an InsP_3 and Ins(1,3,4,5)P_4 has been found for responses in cells from mouse lacrimal glands (A. P. Morris *et al.* 1987) and *Aplysia* neurons

(L. Kaczmarek, personal communication). Spät *et al.* (1987) have observed an additive effect of Ins(1,3,4,5)P_4 on Ins(1,4,5)P_3-induced Ca^{2+} release from a pituitary membrane preparation. The data in the first of these studies (A. P. Morris *et al.* 1987) are entirely consistent with the notion that Ca^{2+} entry is controlled by the two inositol phosphates acting in concert, because the effects of the InsP_3 and InsP_4 together are stable over many minutes, and over this period are entirely dependent on extracellular Ca^{2+}. However, if, as discussed below, acetylcholine is exerting its effects on the cells through inositol phosphates, then these data also suggest a synergism between the two inositol phosphates on intracellular mobilization (because, in the short term, acetylcholine's effects are independent of extracellular calcium and are not mimicked by perfusion of the cells with InsP_3 alone (A. P. Morris *et al.* 1987)). Moreover, in the experiments on *Aplysia* it appears that Ins(1,3,4,5)P_4 may also be in part modulating the mobilization of intracellular Ca^{2+} by Ins(1,4,5)P_3, as, indeed, it might have been in the sea urchin eggs (Crossley *et al.* 1988). This interpretation is not contrary to our suggestion that InsP_4 regulates Ca^{2+} entry from outside the cell (Irvine & Moor 1986; A. P. Morris *et al.* 1987). It can rather be considered as an extension of it; several observations provide a framework on which to build a more general scheme for the action of InsP_4.

The recent work of Dawson's and Gill's groups (Dawson *et al.* 1987; Mullaney *et al.* 1987) has demonstrated a phenomenon in permeabilized cells and in membrane fractions, which is most easily interpreted by suggesting that GTP is controlling the passage of Ca^{2+} between different intracellular compartments (possibly through a channel analogous to that found in gap junctions). This concept is very similar to the proposal of a link between the plasma membrane and the endoplasmic reticulum (Putney 1986; Irvine & Moor 1987; Irvine 1987), and it is therefore most tempting to postulate that the two phenomena are one and the same. It is more likely that, *in vivo*, some other factor is responsible for the phenomenon, the presence of GTP being obligatory rather than serving to control the process. If we propose that Ins(1,3,4,5)P_4 is this controlling factor (figure 1), then we have the general concept of Ins(1,3,4,5)P_4 controlling the size of the Ca^{2+}-pool responsive to Ins(1,4,5)P_3, the possible regulation of Ca^{2+}-entry through the plasma membrane (Irvine & Moor 1986, 1987; A. P. Morris *et al.* 1987) being just one facet of this more general function of Ins(1,3,4,5)P_4 (figure 1).

It is relevant to this discussion to consider also the experiments of Krause & Lew (1987) and of Henne *et al.* (1987) which suggest that the Ca^{2+}-pumping activity in the endomembrane system can to some extent be physically separated from the Ins(1,4,5)P_3-sensitive components. Volpe *et al.* (1988) have provided data which take this idea a stage further, and have proposed a cellular organelle discrete from the endoplasmic reticulum, the calciosome, which is predominantly responsible for calcium homeostasis in the cell. Given the data of Somlyo *et al.* (1986), which show the endoplasmic reticulum to be a major store of Ca^{2+} in the cell, we think that the calciosome concept is still consistent with a spatial separation of the majority of intracellularly stored Ca^{2+} from that subcomponent which is responsive to Ins(1,4,5)P_3; our suggestion is that Ins(1,3,4,5)P_4 serves to unite functionally these two Ca^{2+} pools.

At one extreme, one could therefore suggest that if the Ins(1,4,5)P_3-sensitive pool were very small, then Ins(1,4,5)P_3 would be almost completely dependent on Ins(1,3,4,5)P_4 for mobilizing intracellular Ca^{2+}. The ability of Ins(2,4,5)P_3 to mobilize a large amount of Ca^{2+} on its own in some cells (e.g. *Xenopus* eggs, W. Busa, personal communication; some sea urchin eggs, see above) might appear to argue against such an idea. However, we cannot ignore the possibility that the activation by Ca^{2+} of Ins(1,4,5)P_3-3-kinase (discussed below) may be critical in intact

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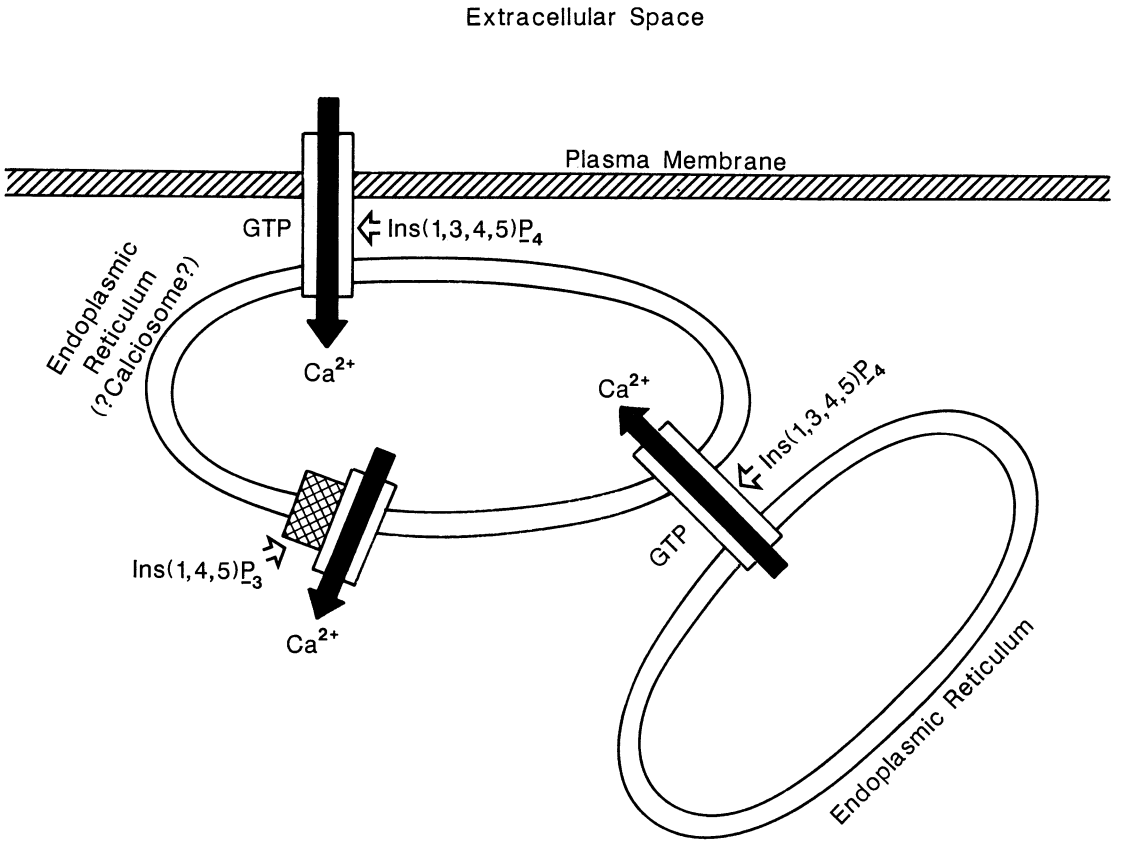

FIGURE 1. Schematic depiction of proposed action of Ins(1,3,4,5)P_4. This diagram is an expansion on the scheme of Irvine & Moor (1987), and depicts the concept that Ins(1,4,5)P_3 releases Ca^{2+} from an intracellular store (the endoplasmic reticulum, or perhaps a specific part of it (see, for example, Krause & Lew 1987; Henne *et al.* 1987; Volpe *et al.* 1988)), and that the function of Ins(1,3,4,5)P_4 is to control Ca^{2+} entry into this store. The Ca^{2+} may come either from parts of the endoplasmic reticulum which pump and store Ca^{2+} but do not respond to Ins(1,4,5)P_3 (see, for example, Henne *et al.* 1987), or possibly from outside the cell (Irvine & Moor 1986, 1987); we suggest that Ins(1,3,4,5)P_4 does this by controlling the formation of GTP-dependent Ca^{2+}-carrying junctions between membranes (see also Mullaney *et al.* 1987).

cells, i.e. that at resting Ca^{2+} levels the kinase is inactive. If that were true, then whether cells respond to injection of Ins(2,4,5)P_3, may be merely a reflection of higher resting amounts of Ins(1,4,5)P_3 in those cells that do; in such cells a small pulse of Ca^{2+} released by Ins(2,4,5)P_3 could possibly cause formation of sufficient Ins(1,3,4,5)P_4 to synergize with the Ins(2,4,5)P_3.

Before leaving the discussion of these possible roles of Ins(1,3,4,5)P_4, it is pertinent to ask whether it could ever act entirely on its own to regulate cellular calcium homeostasis, i.e. does it serve only as a modulator of the action of Ins(1,4,5)P_3? Is it possible for the cell to regulate specifically the amount of Ins(1,3,4,5)P_4, and to bypass the effect on cytoplasmic calcium ion concentrations which results from regulating the amount of Ins(1,4,5)P_3? In cells from mouse lacrimal glands, acetylcholine activates Ca^{2+}-controlled K^+ channels (A. P. Morris *et al.* 1987) even when Ins(1,4,5)P_3 perfused into the same cells does not. The most likely explanation for the effect of acetylcholine is that it causes the stimulated production of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, and if that is so, then it could be that Ins(1,4,5)P_3 kinase is stimulated by receptor activation. If we consider the implications of such a regulation, were it to exist, it

introduces the possibility of the amounts of Ins(1,3,4,5)P_4 increasing with no change (or a decrease?) in that of Ins(1,4,5)P_3. The result of this, drawing on the concepts presented in figure 1, would be a lack of an increase in cytosolic Ca^{2+}, with a redistribution of Ca^{2+} within the subcellular compartments, some of which may respond to Ca^{2+}. For instance, both mitochondria and nuclei (see, for example, Shore & Tata 1977) can be seen to be closely associated with the endoplasmic reticulum; both of these organelles are believed to control their Ca^{2+} homeostasis, and to respond to changes in concentrations of Ca^{2+} within them (see, for example, Denton & McCormack 1986; White 1985; Williams *et al.* 1987). Thus the idea emerges of transfer of Ca^{2+} between compartments to cause marked physiological effects, but with no change in cytosolic Ca^{2+}. Could this be a subtle and important role for Ins(1,3,4,5)P_4?

3. Complexities in the metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4

(*a*) *Regulation of* Ins(1,4,5)P_3-3-kinase

In the light of the probable function of Ins(1,3,4,5)P_4, regulation of Ins(1,4,5)P_3-3-kinase is an interesting facet of cellular Ca^{2+} homoeostasis. The stimulation of this enzyme by Ca^{2+} in whole cells and in homogenates was first shown in insulinoma cells by Biden & Wollheim (1986), and was further demonstrated in other tissues by, for example, Rossier *et al.* (1986), Lew *et al.* (1986), Zilberman *et al.* (1987) and Imboden & Pattison (1987). The regulation by Ca^{2+} was shown to be mediated by calmodulin (Biden *et al.* 1986; A. J. Morris *et al.* 1987; Yamaguchi *et al.* 1987; Kimura *et al.* 1987) with the second example (in turkey erythrocytes) being particularly interesting in that a membrane-bound activity was studied. Ins(1,4,5)P_3-3-kinase is predominantly soluble (see, for example, Irvine *et al.* 1986*a*; Hansen *et al.* 1986; Biden & Wollheim 1986), but if some is membrane-bound in all cells then there are interesting possibilities for regulation of the enzyme either by receptors or protein kinase-C (Imboden & Pattison 1987) (see §2).

In some tissues (e.g. brain (Irvine *et al.* 1986*a*; A. J. Morris *et al.* 1987)), Ins(1,4,5)P_3-3-kinase is not affected by Ca^{2+}, at least in preparations from a tissue homogenate. Whether there may be some level of calmodulin regulation that is destroyed by the homogenization, is not certain. It is also possible that pH may have a role to play in the regulation of Ins(1,4,5)P_3-3-kinase (Irvine *et al.* 1986*a*) because the enzyme shows a sharp cut-off in activity (*in vitro*) below pH 7, though the physiological significance of this is only speculation at the moment. Finally, Batty & Nahorski (1987) have presented evidence that Ins(1,4,5)P_3-3-kinase in rat brain may be either directly or indirectly inhibited by low concentrations of Li^+. The implications of this for the interpretation of the therapeutic effects of Li^+ on the brain are obvious (Batty & Nahorski 1987). In summary, it appears that Ins(1,4,5)P_3-3-kinase is stimulated by Ca^{2+} and possibly by other mechanisms, and we suggest that it is likely that this is a feed-forward mechanism to promote Ins(1,3,4,5)P_4 production and hence recruit more Ca^{2+} for Ins(1,4,5)P_3 to mobilize (as discussed in §2).

(*b*) *Possible functions of* Ins(1,3,4)P_3

Teleologically, one can argue against a messenger molecule being derived (obligatorily) from another messenger molecule with an entirely different function (compare this with the apparently close functional relation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4). However, the

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experiences of the past few years have made us wary of predicting too much, and Ins(1,3,4)P_3 may yet prove to be more than an inactive breakdown product.

Originally, a long-term function for Ins(1,3,4)P_3 (e.g. regulation of gene transcription or cell division) was suggested (Berridge & Irvine 1984). However, injection of Ins(1,3,4)P_3 into sheep oocytes has no detectable effect on patterns of protein synthesis (R.M.M. and R.F.I., unpublished results), and injection into fibroblasts has no effect on transcription of gene *J*, one of the c-*myc* family of genes induced by PDGF (C. D. Stiles and R. F. I., unpublished results). Despite these two clear negative results, this idea still remains open. In assessing all negative results from injection of inositol phosphates, it is crucial to remember that on its own Ins(1,3,4,5)P_4 is also apparently inert, even in cells in which it has such potent effects with an InsP_3 present. Without the discovery of this synergism we would probably still consider Ins(1,3,4,5)P_4 to be biologically inactive; thus if another inositol phosphate gives a negative answer it may be because we are not asking exactly the right question.

Higashida & Brown (1986) and Tertoolen *et al.* (1987) have observed electrical effects (depolarization of NG108 and NIE-115 cells, respectively) caused by pressure-injection of Ins(1,3,4)P_3. In the NG108 cells, however, these effects were also invoked by Ins(1,3,4,5)P_4 (Higashida & Brown 1986) and can also be induced by inorganic phosphate (D. A. Brown, personal communication) so that, in the opinion of those workers, these effects are of doubtful physiological significance. In the NIE-115 cells, Ins(1,3,4,5)P_4 did not mimic the effect of Ins(1,3,4)P_3 (Tertoolen *et al.* 1987); the specificity is still open to question in that if Ins(1,4,5)P_3 had had the same effect, it would have been masked by the hyperpolarization induced by the Ca^{2+}-mobilization which also occurred. Also, only about 50% of the cells respond to injection of Ins(1,3,4)P_3 (Tertoolen *et al.* 1987). Nevertheless, the effect is there and is not entirely a non-specific effect of an anionic molecule, so there remains the possibility of Ins(1,3,4)P_3 causing a long-term (over a period of a few minutes) depolarization of neurons.

In some cells Ins(1,3,4)P_3 may also mobilise Ca^{2+}. When it does, it is not very potent (e.g. 30 times less so than Ins(1,4,5)P_3 (Irvine *et al.* 1986*b*)) so we advocate that only with 'HPLC-pure' (high-performance liquid chromatography-pure) Ins(1,3,4)P_3 should one draw firm conclusions. Nevertheless, Ins(1,3,4)P_3 (not HPLC-pure) is about half as potent as Ins(1,4,5)P_3 is in depolarizing *Limulus* photoreceptors (J. E. Brown, personal communication) and it may be that in this tissue the resistance of Ins(1,3,4)P_3 to Ins(1,4,5)P_3-5-phosphatase (Irvine *et al.* 1984) may help magnify its potency. By contrast, in brain membranes Ins(1,3,4)P_3 does not compete for Ins(1,4,5)P_3-binding sites (Willcocks *et al.* 1987) and in the hands of the same group (S. R. Nahorski, personal communication) it also does not mobilize Ca^{2+} from Swiss mouse 3T3 cells. Also, Ins(1,3,4)P_3 is completely incapable of activating eggs of *Lytechinus pictus* (Crossley *et al.* 1988) and does not mobilize Ca^{2+} from rat liver microsomes even at 100 times the maximally active dose of Ins(1,4,5)P_3 (R. Cullen & A. P. Dawson, personal communication). Overall, the evidence in most tissues does not favour the idea that Ins(1,3,4)P_3 mobilizes Ca^{2+}; the issue is still open, however, and given the large amounts of Ins(1,3,4)P_3 that can be attained in some tissues (see previous section) it is at least possible that it may sometimes serve a function as a 'long-term Ins(1,4,5)P_3'.

In conclusion, the presence of Ins(1,3,4)P_3 remains enigmatic as regards function. On balance, the evidence is against a physiological role *in vivo*, but the various effects documented above may be pointing to a role in some tissues, under certain conditions.

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(*c*) *Metabolism of* Ins(1,4)P_2 *and* Ins(1,3,4)P_3

It is generally agreed that the first step in the catabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 is by removal of the 5-phosphate (Downes *et al.* 1982; Batty *et al.* 1985) to yield Ins(1,4,)P_2 and Ins(1,3,4)P_3, respectively. Evidence has been presented for the formation of Ins(4,5)P_2 and Ins(5)P (Hughes & Drummond 1988), so even this statement may be an over-simplification. Nevertheless, the predominant route is almost certainly by 5-phosphate removal, which is likely to be catalysed by the same enzyme (Connolly *et al.* 1987), although enzymes for specific substrates, in addition to this common enzyme in platelets, have not been ruled out. The prolonging by Ins(1,3,4,5)P_4 of the Ins(1,4,5)P_3-induced Ca^{2+} transient in permeabilized hepatocytes is very likely to be by inhibition of the hydrolysis of Ins(1,4,5)P_3 (Joseph *et al.* 1987) implying that, in these cells at least, both substrates are being hydrolysed by the same enzyme(s). It seems highly unlikely that inhibition of the hydrolysis of Ins(1,4,5)P_3 by such competition is responsible for the biological effects of Ins(1,3,4,5)P_4 as reported (Irvine & Moor 1986; A. P. Morris *et al.* 1987); in the first instance the 'potency' of Ins(2,4,5)P_3 was increased over at least two orders of magnitude by Ins(1,3,4,5)P_4, and in the second, Ins(1,4,5)P_3 continuously perfused into mouse lacrimal glands at 100 μM is entirely ineffective in activating the Ca^{2+}-dependent K^+ current, whereas at 10 μM it synergizes to give a full effect with Ins(1,3,4,5)P_4 (A. P. Morris *et al.* 1987). Inositol phosphate-5-phosphatase has been suggested to be regulated by protein kinase C (Connolly *et al.* 1986) and it may also be regulated by Ca^{2+} in other tissues (see Irvine (1988) for review).

The subsequent metabolism of the Ins(1,4)P_2 and Ins(1,3,4)P_3 formed by the 5-phosphatase has been found to be complex and appears to differ considerably between tissues. Ins(1,4)P_2 can be metabolized *in vitro* to either Ins(1)P (Storey *et al.* 1984; Takimoto *et al.* 1987) or Ins(4)P (Inhorn *et al.* 1987; Ackermann *et al.* 1987; Moyer *et al.* 1987; Delvaux *et al.* 1987) by enzymes which may (Storey *et al.* 1984) or may not (Ackermann *et al.* 1987) be of different sensitivity to Li^+. One problem with these studies is to relate cell homogenate data to the whole cell (i.e. is one a true reflection of the other?); recent studies on the metabolism of Ins(1,4)P_2 microinjected into *Xenopus* oocytes (M. J. Berridge, R. F. I. and K. A. W., unpublished results) support Ins(4)P as the predominant product of Ins(1,4)P_2 catabolism in at least that particular cell. At present, the majority of evidence favours a route of cellular metabolism of Ins(1,4)P_2 to Ins(4)P in most cells, and it is not clear at present whether the accumulation of Ins(1)P reflects (*a*) the alternative route of Ins(1,4)P_2 metabolism, (*b*) a contribution from Ins(1,3,4)P_3 (see below and figure 2), or (*c*) PtdIns hydrolysis.

The catabolism of Ins(1,3,4)P_3 is even more complex. The occurrence of Ins(3,4)P_2 in stimulated cells (Irvine *et al.* 1987; Dillon *et al.* 1987*a*, *b*; Hughes & Drummond 1987) shows that at least part of the catabolism involves the removal of the 1-phosphate (see also Shears *et al.* 1987*a*; Inhorn *et al.* 1987; Erneux *et al.* 1987). Considerable insight into the enzymology of this catabolism has been gained from recent work in Majerus's group (Inhorn *et al.* 1987; Inhorn & Majerus 1987; Bansal *et al.* 1987). The enzyme that removes the 1-phosphate from Ins(1,3,4)P_3 is probably the same as that that removes the same phosphate from Ins(1,4)P_2 (Inhorn & Majerus 1987). The inhibition of the enzyme by Li^+ is uncompetitive (Inhorn & Majerus 1987) by which those authors have explained the observation of Shears *et al.* (1987*a*) that only at high Ins(1,4)P_2 levels does Li^+ inhibit Ins(1,3,4)P_3 hydrolysis. That this inositol polyphosphate-1-phosphatase will not hydrolyse Ins(1,4,5)P_3 in an analogous reaction (Inhorn

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& Majerus 1987), suggests that it is not a non-specific phosphatase masquerading as an inositol polyphosphate phosphatase, but that it is probably there to catalyse specifically the breakdown of Ins(1,3,4)P_3 and Ins(1,4)P_2.

This is not the only route of Ins(1,3,4)P_3 hydrolysis, Ins(1,3)P_2 having been demonstrated in GH_4 cells (Irvine *et al.* 1987). In cultured human umbilical vein endothelial cells, the predominant inositol bisphosphates to accumulate in the presence of Li^+ are Ins(1,4)P_2 and (probably) Ins(1,3)P_2 (Pollock *et al.* 1988). Bansal *et al.* (1987) have shown that an enzyme exists in calf brain which removes the 4-phosphate from Ins(1,3,4)P_3. This is an Mg^{2+}-independent, Li^+-insensitive enzyme that also hydrolyses Ins(3,4)P_2 to Ins(3)P (see also Dillon *et al.* (1987*b*); it would be interesting to know if this is also the enzyme responsible for the removal of the 4-phosphate from Ins(1,4)P_2, as studied by Takimoto *et al.* (1987) although the Li^+-sensitivity of the latter enzyme argues against this). The Ins(1,3)P_2 formed by the activity of this enzyme is probably further degraded to Ins(1)P (Bansal *et al.* 1987). These routes of catabolism are summarized in figure 2.

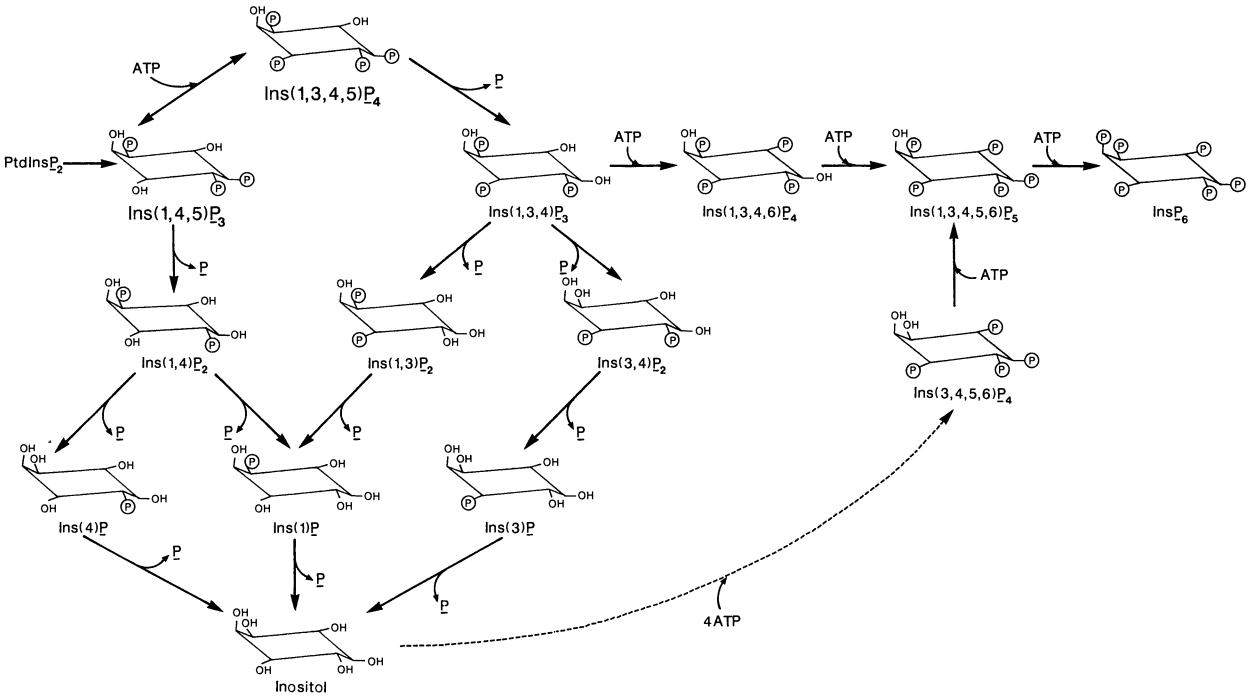

FIGURE 2. Inositol phosphate metabolism. This figure summarizes our current understanding of the metabolism of inositol phosphates derived from Ins(1,4,5)P_3. Note that unless analysed by the very difficult analytical technique of chiral gas–liquid chromatography (Sherman *et al.* 1981), Ins(3)P and Ins(1)P are indistinguishable because they are an enantiomeric pair; thus the product of Ins(1,3,4)P_3 metabolism by the route in which the 4-phosphate is removed first, will appear on routine analysis as 'Ins(1)P'.

If we regard Ins(1,3,4)P_3 as having no function (see above) then it is pertinent to enquire why its catabolism is so complex, and why its metabolism may vary between tissues. Does this apparently tissue-dependent, complex metabolism of Ins(1,3,4)P_3 imply that at least one of the inositol bisphosphate products has an, as yet undisclosed, physiological role? The possibility is an exciting one to entertain and at the very least this complexity is a sobering reminder of how little we know.

There is, however, an alternative way of viewing this complexity, and that is to regard it simply as an inevitable consequence of the enzymology of the hydrolytic pathways, and we should not therefore read too much into it. In figure 3 we have presented most of the information of figure 2, in a simplified form, emphasizing that there are two specific inositol polyphosphate phosphatases involved once the 5-phosphatase has generated the Ins(1,4)P_2 and

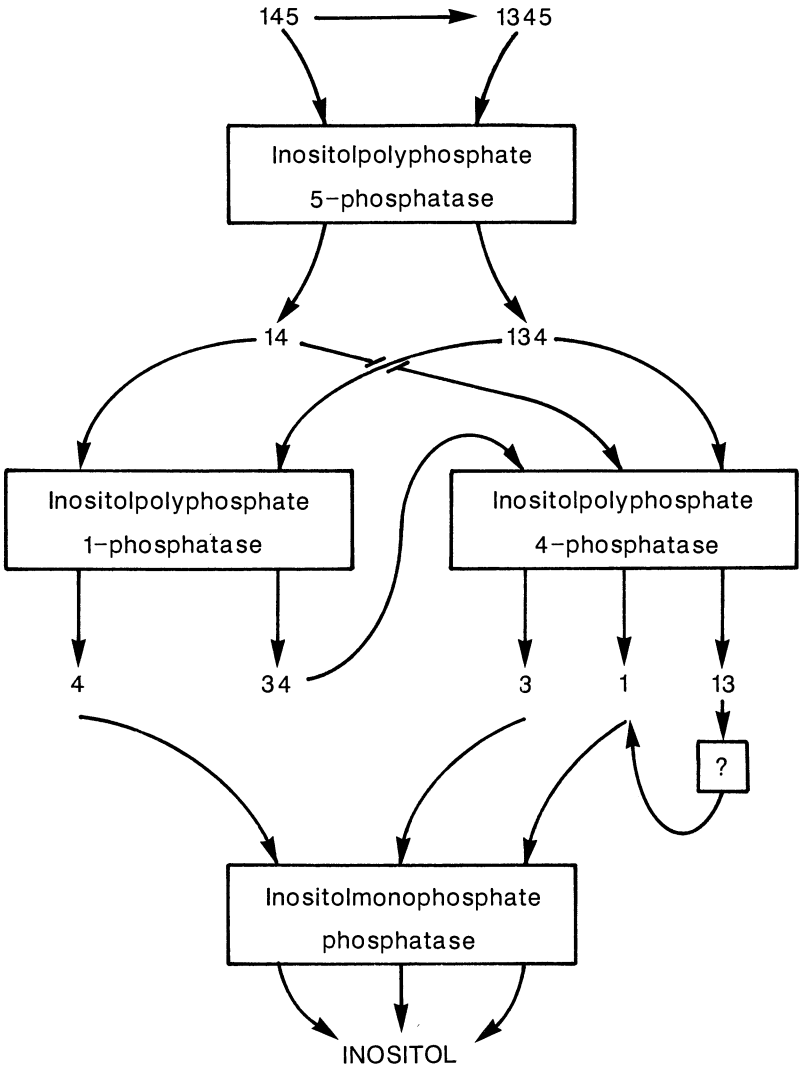

FIGURE 3. Metabolism of Ins(1,4)P_2 and Ins(1,3,4)P_3. This figure is a simplified version of part of figure 2, but redrawn to present what we believe to be a likely explanation for the labyrinth of inositol phosphate metabolism by emphasizing which enzymes possibly catalyse the various reactions.

Ins(1,3,4)P_3, and that both of these enzymes can hydrolyse several substrates. (Note that we do not know the identity of the phosphatase involved in the 3-dephosphorylation of Ins(1,3)P_2 which has been described by Bansal *et al.* (1987).) If the relative activities of these two inositol polyphosphate phosphatases as well as the relative rates of formation of Ins(1,4)P_2 and Ins(1,3,4)P_3, vary between tissues, then it is not at all surprising that marked differences occur in steady-state levels of the various intermediates. This is not without its own significance in

that, for example, accumulation of Ins(1,4)P_2 could inhibit hydrolysis of Ins(1,3,4)P_3 (see Shears *et al.* 1986), and thus the sharing of enzymes between the hydrolytic pathways (figures 2 and 3) may lead to cross-talk between them. It is interesting to note that neither Ins(1,4,5)P_3 nor Ins(1,3,4,5)P_4 are hydrolysed by the 1- or 4-inositol polyphosphate phosphatases (Bansal *et al.* 1987; Inhorn *et al.* 1987) and this prompts the thought that the 5-hydroxyl, para to the axial hydroxyl (number 2), may be the recognition or orientation site for these two enzymes.

A recent observation made in three laboratories has provoked a whole new range of questions about the metabolism and function of Ins(1,3,4)P_3; this is the discovery of a distinct kinase that phosphorylates Ins(1,3,4)P_3 to Ins(1,3,4,6)P_4 (Shears *et al.* 1987*b*; Balla *et al.* 1987*a*, *b*; L. Stephens, P. T. Hawkins and C. P. Downes, unpublished results). The structure of Ins(1,3,4,6)P_4 is as yet assigned on indirect evidence (Shears *et al.* 1987*b*; Balla *et al.* 1987*b*), though it is very likely that this assignation is correct. Unpublished observations from all three groups suggest that Ins(1,3,4,6)P_4 is further phosphorylated to an InsP_5, and current data (L. Stephens, personal communication) show that this InsP_5 is Ins(1,3,4,5,6)P_5.

The functions of the inositol phosphates in this unexpected metabolic route are unknown. From a space-filling model of Ins(1,3,4,6)P_4, it does not seem unreasonable to predict that it might mobilize Ca^{2+} (despite its extra phosphate, it fits well into the spatial distribution of phosphate groups formed by Ins(1,4,5)P_3 and Ins(2,4,5)P_3). Ins(1,3,4,5,6)P_5 can mimic the effect of Ins(1,3,4,5)P_4 in eggs of *Lytechinus variegatus* (Irvine & Moor (1987) and see §4), and the putative Ins(1,3,4,5)P_4-'receptor' shows an apparent affinity for Ins(1,3,4,5,6)P_5 that is very similar to that of Ins(1,3,4,5)P_4 (Bradford & Irvine 1987). Ins(1,3,4,6)P_4 and Ins(1,3,4,5,6)P_5 might therefore be the 'long-term' equivalents of Ins(1,4,5)P_3 and Ins-(1,3,4,5)P_4, respectively. However, until we know more about the physiological activity of Ins(1,3,4,6)P_4, this present addition to receptor-generated inositol phosphate metabolism remains something of a mystery.

In conclusion, two extreme viewpoints can be advanced to explain why the metabolism of receptor-generated inositol phosphates is so complex (figure 2). At one extreme one could argue that the complexity reflects functions for some of these compounds of which we have no hint at the moment. The other way of looking at figure 2, which we have also discussed, is that the extra complexity is all ultimately an indirect result of the formation and function of Ins(1,3,4,5)P_4, i.e. that a system that produces two second messengers with closely related functions, will inevitably have a complex catabolic metabolism. Perhaps the true explanation will lie somewhere between these two extremes.

4. Inositol pentakis- and hexakisphosphates

These compounds have for years been known to exist in plants and in avian erythrocytes (see, for example, Cosgrove 1980), and so their discovery in mammalian cells and some other animal tissues (see, for example, Heslop *et al.* 1985; Morgan *et al.* 1987; Tilly *et al.* 1987; Jackson *et al.* 1987; Stewart *et al.* 1987) was perhaps, with the benefit of hindsight, not surprising. The route of synthesis of InsP_5 in erythrocytes has been the subject of elegant investigation by L. Stephens and his colleagues in C. P. Downes's laboratory (for further details see Stephens *et al.* 1988*a*, *b*). The precursor to Ins(1,3,4,5,6)P_5 is probably Ins(3,4,5,6)P_4 (a compound tentatively identified previously by Johnson & Tate (1969)), but at present the

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precursor to this InsP_4 is not known. A kinase that will convert Ins(3,4,5,6)P_4 to an Ins(1,3,4,5,6)P_5 has been detected by Stephens *et al.* (1988*b*) in the homogenates of a number of animal tissues. In the same experiments Ins(1,3,4,5)P_4 was not phosphorylated at all (Stephens *et al.* 1988*b*), which clearly points to the metabolic separation of the InsP_5/InsP_6 synthesis pathway from the receptor-generated InsP_3/InsP_4 pathway of Irvine *et al.* (1986*a*) (though one should not forget that Ins(1,3,4)P_3 phosphorylation (above) may provide an interconnection).

The separation of these two pathways prompts us to question where in the cell InsP_5 and InsP_6 are synthesized. Two observations suggest a separate sub-cellular compartment distinct from the cytosol. Firstly, there is the experimental observation that Ins(1,3,4,5,6)P_5 is able to mimic the physiological effects of Ins(1,3,4,5)P_4 in sea urchin eggs (Irvine & Moor 1987) and sheep oocytes (B. G. Cran, R.M.M. & R.F.I., unpublished results) and its binding to HL-60 membranes (Bradford & Irvine 1987). We have argued teleologically from this (Irvine & Moor 1987) that it is unlikely that large amounts of InsP_5 (as found for example by Tilly *et al.* (1987) and Jackson *et al.* (1987)) will be present in the same cytosol in which metabolism of Ins(1,3,4,5)P_4 is being closely controlled. This reasoning is not inconsistent with the argument advanced above that Ins(1,3,4,5,6)P_5 formed from Ins(1,3,4)P_3 via Ins(1,3,4,6)P_4, could be a 'long-term' analogue of Ins(1,3,4,5)P_4; in that context the concentration of Ins(1,3,4,5,6)P_5 in the cytosol may be low, and only reach physiologically significant levels while the cell is being stimulated.

While considering the unlikelihood of high resting amounts of biologically active InsP_5 in the cytosol, it is worth drawing attention to the data of Tashjian *et al.* (1987) in which high resting amounts of 'Ins(1,4,5)P_3' were reported in GH_4 cells. The result of these high resting amounts was that only some time after intracellular stores of Ca^{2+} had been maximally mobilized was there a detectable increase in the amount of Ins(1,4,5)P_3. L. R. Stephens (personal communication) has found high amounts of D-Ins(1,4,5)P_3 in turkey erythrocytes, suggesting that it might be an intermediate in the synthesis or degradation of Ins$P_{5\,or\,6}$. We have re-examined the 'Ins(1,4,5)P_3' present in GH_4 cells, and have found that 80–90% is apparently D-Ins(1,4,5)P_3 (based on hydrolysis to Ins(1,4)P_2 by the Ins(1,4,5)P_3-5-phosphatase (R.F.I., K.A.W., A.J. Letcher, M.J. Berridge & A. H. Tashjian, unpublished results)). Is it really possible that a high resting amount of this known second messenger is present in the cytosol, or again, is it compartmentalized elsewhere?

The second line of evidence for discrete compartmentalization of InsP_5 and InsP_6 is from studies on the solubility of inositol phosphates in Mg^{2+}- and Ca^{2+}-containing solutions (J. Rogers, A. Pini, G. Henderson & M. R. Hanley, personal communication). These data infer that Ins(1,3,4,5,6)P_5 at concentrations in excess of 50 μM (which are implied by the data of Tilly *et al.* (1987) and Jackson *et al.* (1987)) or InsP_6 at a concentration of 700 μM (Martin *et al.* 1987), are not likely to form stable solutions in the cytosol, i.e. InsP_5 and InsP_6 at these amounts may have to be sequestered in a compartment that has a low divalent-cation concentration or an acidic environment.

Whatever the localization of InsP_5 and InsP_6, their functions are even more obscure. InsP_5 and InsP_6 could serve the same function as they do in avian erythrocytes, i.e. modulation of the quaternary structure of a protein (see Isaacks *et al.* 1977; Teisseire *et al.* 1987). It could be that their action is extracellular and that they serve as neurotransmitters or mediators or both between cells as suggested by Vallejo *et al.* (1987), though the electrophysiological effects seen

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by these workers could be due to the artefactual removal of Ca^{2+} or Mg^{2+} by the InsP_5 and InsP_6 (see Hanley, this symposium).

An alternative possibility (not mutually exclusive to the proposed extracellular role) that we would like to put forward, is that their function is the same as that ascribed to InsP_6 in higher plants: that of phosphate storage. There is evidence that inorganic phosphate is stored near the plasma membrane in pancreatic B cells, and that when these cells are stimulated to secrete, this phosphate is rapidly secreted along with insulin (Freinkel *et al.* 1978). The reason for this phosphate flush' is not known, but it does point to the possibility that cells (especially secretory cells) may need to have a rapidly accessible phosphate store. Inorganic phosphate stores (Freinkel *et al.* 1978) must be of a limited size, and a more substantial organic reservoir may be required; InsP_5 and InsP_6 could serve as this reservoir.

Whatever the functions of InsP_5 and InsP_6, their probable universal presence is another aspect of the uses to which evolution has put *myo*-inositol. As discussed above, the discovery of the conversion of Ins(1,3,4)P_3 (a receptor-generated inositol phosphate) to an InsP_5 may link the two inositol polyphosphate pathways; a fascinating time awaits us as we unravel these complications.

5. Cyclic inositol phosphates

After the initial discovery of cyclic inositol phosphate (cycIns(1–2)P) by Dawson *et al.* (1971), and the flurry of activity centred around the possibility that it might be a second messenger (see, for example, Lapetina & Michell 1973), interest in cyclic inositol phosphates subsided into the background. However, the observation by Wilson *et al.* (1985) that hydrolysis of PtdInsP_2 by PtdInsP_2 phosphodiesterase could form cycIns(1–2,4,5)P_3, allied with the physiological activity of cycIns(1–2,4,5)P_3 (Wilson *et al.* 1985; Irvine *et al.* 1986*b*) led to the suggestion that cycIns(1–2,4,5)P_3 is a second messenger in its own right (see Majerus *et al.* (1986) for review).

There is no doubting the ability of cycIns(1–2,4,5)P_3 to mobilize Ca^{2+} (Wilson *et al.* 1985; Irvine *et al.* 1986*b*). (This is a pertinent point at which to note that earlier (Irvine & Moor 1986) we quoted a personal communication from Swann & Whitaker that cycIns(1–2,4,5)P_3 does not activate sea urchin eggs; this was an error in that cycIns(1–2)P was the compound tested (M. Whitaker, personal communication).) However, the key questions as to the function of cycIns(1–2,4,5)P_3 are; does it occur naturally and is its formation stimulated by receptor occupation? At present the answer to these questions is equivocal. The only evidence presented that cycIns(1–2,4,5)P_3 is a major component of the InsP_3 fraction of a stimulated tissue is in platelets stimulated by thrombin (Ishii *et al.* 1986); in those experiments, because of the use of ^{32}P to label the tissue, there are enormous difficulties in determining whether only inositol phosphates are being examined (see Irvine (1988) for further discussion). In addition Tarver *et al.* (1987) have re-examined platelets by measuring the mass of inositol phosphates, and they conclude that cycIns(1–2,4,5)P_3 only represents a minority of the InsP_3 even after prolonged stimulation.

In tissues labelled with [^{3}H]*myo*-inositol, where less equivocal results have been obtained, cycIns(1–2,4,5)P_3 is not found after short stimulation times, it only accumulates over a long time, and even then it is still a minority (see, for example, Sekar *et al.* 1987; Hawkins *et al.* 1987). CycIns(1–2,4,5)P_3 is a poor substrate for Ins(1,4,5)P_3-5-phosphatase (Connolly *et al.*

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1986, 1987; Hawkins *et al.* 1987) and an extremely poor substrate for Ins(1,4,5)P_3-3-kinase (Connolly *et al.* 1987; Hawkins *et al.* 1987), so a slow accumulation might be expected if it is formed at a slow rate when a tissue is stimulated. In conclusion, given the physiological activity of cycIns(1–2,4,5)P_3 and its slower metabolism, it is possible that it could act as a 'long-term Ins(1,4,5)P_3' in the manner suggested for Ins(1,3,4)P_3 or Ins(1,3,4,6)P_4 above. Other than that, at present its presence is of doubtful physiological significance.

6. Conclusions

It will be apparent from the discussion above that the proliferation of inositol phosphates is not over yet. If we consider both the possibility of a conjugated inositol phosphate being the mediator for some of insulin's action (see Saltiel, this symposium) and the demonstration of a function of inositol lipids as protein anchors that has emerged in recent years (see Low 1987), then we must be impressed with the variety of uses to which *myo*-inositol has been put by the cell. The function of Ins(1,4,5)P_3 as a second messenger can now be regarded as being firmly established, and the putative third second messenger derived from polyphosphoinositol lipids, Ins(1,3,4,5)P_4, is rapidly gaining respectability. But beyond that, there is only speculation. Above, we have summarized these speculations and added a few of our own.

As stated in the Introduction, six years ago in a volume similar to this, Michell *et al.* (1981) made the first concrete suggestion that PtdIns(4,5)P_2 may be the primary substrate for receptor-activated inositol lipid hydrolysis. If progress in the next six years is anywhere near that in the past six (during which nearly all of the observations discussed above have emerged), then by the early 1990s we will have a much better idea of (*a*) why there are so many inositol phosphates and (*b*) just how many there are. Although the possibility of phosphates of inositols other than *myo*-inositol is something we cannot ignore (see Sherman *et al.* 1978), for the moment the laws of chemistry, mercifully, restrict the number of inositol phosphates to sixty-three.

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We are grateful to our many colleagues, especially those in Cambridge and Welwyn, and Don Gill and Jim Putney, for communicating unpublished results to us, and for numerous discussions.

This year (of writing) is the hundredth anniversary of the demonstration by Maquenne (1887*a*, *b*, *c*) that inositol is a cyclohexanol; as this volume summarizes the enormous biological importance of *myo*-inositol, it seems appropriate to draw attention to Maquenne's classic work and to dedicate this review to a recognition of his pioneering studies.

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Discussion

C. P. Downes (*Smith Kline & French Research Ltd, Welwyn, U.K.*). In the InsP_4 binding studies I was concerned by the shallow displacement curves for all the compounds examined, including Ins(1,3,4,5)P_4. Is this consistent with the idea that the ligand is binding to a single site?

R. F. Irvine. By 'shallow', I assume Dr Downes means a competition curve that extends from total binding to what is defined as non-specific binding, over more than three orders of magnitude of concentration. We do not have enough data either to be able to make quantitative statements or to interpret our data in terms of binding models, and there are several theoretical explanations, none of which we would care to propose at this time, to account for why some of the competition curves do extend over slightly more than this range. As we discuss in the paper reporting these data (Bradford & Irvine 1987), there are several intracellular proteins that might be expected to bind Ins(1,3,4,5)P_4, and it may therefore be that we have to be lucky to some extent to find the cells and the conditions such that the Ins(1,3,4,5)P_4 'receptor' predominates. Even if we have been sufficiently lucky with HL-60 cells, some contribution from other Ins(1,3,4,5)P_4-binding proteins cannot be ruled out. These proteins would have to have binding affinities about a 100-fold more or less than that of the 'receptor' to result in a 'shallow' competition curve. These particular experiments were not intended as a detailed quantitative analysis, but we do think that they define the basic pharmacology of a binding site which, as we discuss, has characteristics consistent with it being the putative Ins(1,3,4,5)P_4 'receptor'.

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A. Spät (*Semmelweis University Medical School, Budapest, Hungary*). Potentiation of Ins(1,4,5)P_3-induced Ca^{2+} release by Ins(1,3,4,5)P_4 in bovine pituitary microsomes and a rat liver particulate fraction confirms Dr Irvine's observations on the synergism of the two inositol phosphates. However, our preliminary data also suggest that Ins(1,3,4,5)P_4 may inhibit the reuptake of Ca^{2+} by the microsomal vesicles. Dr Irvine's evidence that Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 induce the opening of Ca-dependent K^+ channels by enhancing the entry of extracellular Ca^{2+} is based on the observation that Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are without effect in the absence of extracellular Ca^{2+}. One may, however, also consider the possibility that the removal of extracellular Ca^{2+} results in the elevation of cytoplasmic Na^{2+} (by the action of Na^+ or Ca^{2+} antiporter) which, in turn, may somehow antagonize the synergistic action of IP_3 and IP_4 exerted on an intracellular Ca^{2+} store.

R. F. Irvine. I agree that Ins(1,3,4,5)P_4 may be synergizing with Ins(1,4,5)P_3 to mobilize intracellular Ca^{2+}, and this possibility is one that we discuss in some detail above. Our experiments cannot, strictly speaking, rule out the possibility that Ins(1,3,4,5)P_4 is inhibiting Ca^{2+} efflux, but I think that it is a very unlikely explanation for such a dramatic synergism as that observed in sea urchin eggs (Irvine & Moor 1986) and mouse lacrimal glands (A. P. Morris *et al.* 1987). In fact, I can offer an alternative explanation for Dr Spät's preliminary data. If one takes the concept that we have discussed above of Ins(1,3,4,5)P_4 acting to link functionally Ca^{2+} pools, then in the presence of Ins(1,4,5)P_3 some membrane vesicles which were previously (before Ins(1,3,4,5)P_4 addition) insensitive to Ins(1,4,5)P_3, would now (after Ins(1,3,4,5)P_4 addition) be responsive to Ins(1,4,5)P_3. Ca^{2+} re-uptake in these circumstances must be entirely due to Ins(1,4,5)P_3-insensitive vesicles, and as the addition of Ins(1,3,4,5)P_4 has effectively removed some of these (by making them Ins(1,4,5)P_3-sensitive), it will therefore inhibit the re-uptake of Ca^{2+}. Obviously, the development of a large reproducible Ins(1,3,4,5)P_4 response *in vitro* will enable us to answer questions of this sort, and we are working towards that goal now.

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