REVIEW ARTICLE

Metabolism of the inositol phosphates produced upon receptor activation

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INTRODUCTION

It is widely appreciated that in a large variety of cell types, receptor-activated phospholipase C hydrolyses $\text{PtdIns}(4,5)\text{P}_2$ to yield 1,2-diacylglycerol and $\text{Ins}(1,4,5)\text{P}_3$; both products act as intracellular signals, the former by activating protein kinase C and the latter by mobilizing intracellular Ca^{2+} pools (reviewed in [1,2]). The concerted actions of a series of phosphatases and kinases convert $\text{Ins}(1,4,5)\text{P}_3$ to a number of additional inositol phosphates (Fig. 1). This process terminates the signalling activities of $\text{Ins}(1,4,5)\text{P}_3$ and salvages Ins for lipid resynthesis. Additionally, some of the intermediates that are produced may have their own physiological activities; $\text{Ins}(1,3,4,5)\text{P}_4$ may modulate cellular Ca^{2+} fluxes [3–5], whilst InsP_5 and InsP_6 may act as extracellular signals [6]. Thus many laboratories are now studying inositol phosphate metabolism, and their work is summarized in this review.

It is very difficult to analyse the fluxes of individual inositol phosphates in intact cells, for a number of

reasons. The first complication is that $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis is not, potentially, the only receptor-initiated entry point into the metabolic cycle; phosphodiesteric attack upon PtdIns and $\text{PtdIns}4\text{P}$ may yield $\text{Ins}1\text{P}$ and $\text{Ins}(1,4)\text{P}_2$ (see [1,2]). $\text{PtdIns}3\text{P}$ [7] and PtdInsP_3 [tentatively proposed to be $\text{PtdIns}(3,4,5)\text{P}_3$] [8] are two recently identified inositol lipids which, if attacked by phospholipase C, would produce $\text{Ins}(1,3)\text{P}_2$ and $\text{Ins}(1,3,4,5)\text{P}_4$ respectively. Thus we cannot always be sure of the origins of an inositol phosphate in an extract from intact cells.

The study of inositol phosphate metabolism is also complicated by the unexpected variety of inositol phosphate isomers, which are frequently difficult to separate. Indeed, the earliest measurements of cellular $\text{Ins}(1,4,5)\text{P}_3$ levels were overestimates due to contamination by $\text{Ins}(1,3,4)\text{P}_3$. Similarly, many analyses of $\text{Ins}(1,3,4,5)\text{P}_4$ in cells have now to be reassessed with the discovery of further isomers of InsP_4 (see below). The study of multiple isomers of InsP , including enantiomeric

Fig. 1. Inositol phosphate metabolism

Abbreviation: P'ase, phosphatase.

Abbreviations used: PtdIns , phosphatidylinositol; Ins , 1D-*myo*-inositol; P , phosphate; locants are designated where appropriate, with 1:2cyc representing the 1,2-cyclic phosphate; MeInsP_3 , (1-methylphosphoryl)inositol 4,5-bisphosphate.

pairs, is also complex. There is the further complication that PtdIns(4,5) P_2 hydrolysis may also yield a proportion of Ins(1:2cyc,4,5) P_3 as well as Ins(1,4,5) P_3 (Fig. 1). In view of these problems, the data obtained from intact cells that are collated in this review are biased towards the most recent studies, since these have separately analysed larger numbers of metabolites. Of course, the longevity of even some of these conclusions may depend upon the extent of further metabolites which undoubtedly remain to be uncovered. In this respect, it is known that avian erythrocytes and plants contain various inositol phosphates of unknown origins (see [9] for a review). It is beginning to appear that these intermediates may also surface in mammalian cells, although their identities are still unclear, as is the extent to which they are independent from inositol phosphates produced by receptor activation.

INOSITOL PHOSPHATE PHOSPHATASES AND KINASES

Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 5-phosphatase

Akhtar & Abdel-Latif [10], using rabbit iris muscle, were the first to recognize that, following receptor-activated PtdIns(4,5) P_2 hydrolysis, the resultant Ins(1,4,5) P_3 was rapidly dephosphorylated. {Two years earlier the possibility of an active intracellular Ins(1,4,5) P_3 phosphatase had been rejected as being unlikely [11].} The specificity of the Ins(1,4,5) P_3 phosphatase for the 5-phosphate was first demonstrated in erythrocytes [12] and then insect salivary glands [13]. The discovery of the second messenger action of Ins(1,4,5) P_3 [14] led quickly to observations of Mg^{2+} -dependent Ins(1,4,5) P_3 5-phosphatase activity in further cell types [15–20].

The proportion of total cellular 5-phosphatase activity that is membrane-bound has been determined for several cell types, and the results vary from over 70 % [15,17–22], down to 50–60 % [23–26], to 16 % [16]. It is unclear how these differences will affect Ins(1,4,5) P_3 metabolism in the various cells. However, some of the apparent variation in enzyme distribution may be related to the ferocity of the different fractionation techniques that have been employed, and the tenacity with which the enzyme is associated with the membranes. For example in platelets, half of the membrane-bound 5-phosphatase appears to be a loosely attached extrinsic protein since it can be released by 1 M-NaCl [25]. A further, rather neglected, complication is the possibility that in some cases a proportion of the soluble phosphatase activity may be due to non-specific attack by PtdIns(4,5) P_2 5-phosphatase [27,28].

When Ins(1,3,4,5) P_4 was first discovered [29], it was also noted that this compound was a substrate for 5-phosphatase activity in erythrocyte ghosts. Whether a single enzyme catalyses the dephosphorylation of both Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 is a problem best resolved in a purified enzyme preparation. No particle-associated 5-phosphatase activity has so far been subjected to this analysis, but a soluble 5-phosphatase activity has been purified from brain to apparent homogeneity, and this attacked both Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 [21]. These two compounds were also hydrolysed by a soluble 5-phosphatase from platelets that was highly purified (but not to homogeneity; see below) [30]. That the platelet enzyme preparation contained a single 5-phosphatase activity was further indicated by the observation that

Ins(1,4,5) P_3 metabolism was inhibited by Ins(1,3,4,5) P_4 , and the K_i of this effect (0.5 μ M) was similar to the K_m for hydrolysis of Ins(1,3,4,5) P_4 [30]. At least in liver [31], competition of both substrates for a single phosphatase may extend the intracellular half-life of Ins(1,4,5) P_3 and prolong Ca^{2+} mobilization.

Since Ins(1,4) P_2 does not mobilize Ca^{2+} [1], 5-phosphatase plays a vital role in terminating the release of Ca^{2+} by Ins(1,4,5) P_3 . Additionally, if Ins(1,3,4,5) P_4 is involved in regulating Ca^{2+} fluxes in cells [3–5], and Ins(1,3,4) P_3 is either inactive or has very weak control over Ca^{2+} mobilization [43,44,46,59], 5-phosphatase activity will further contribute to the regulation of cellular Ca^{2+} . Some experiments indicated that Ca^{2+} is quite effectively mobilized by Ins(1:2cyc,4,5) P_3 but not by Ins(1:2cyc,4) P_2 [43,47], which suggests that the 5-phosphatase has a further responsibility towards the regulation of cell signalling. However, further work has not confirmed this [45]. Ins(1:2cyc,4,5) P_3 is a relatively poor substrate for the 5-phosphatase [30,32–34], and is not a substrate for the 3-kinase (see below). Indeed, it is the relatively slow metabolism of Ins(1:2cyc,4,5) P_3 that causes it to accumulate in cells [34–36] even when it is only a minor product of receptor-activated PtdIns(4,5) P_2 hydrolysis [33,38,39]. A recent report [34] demonstrates, at least in parotid cells, that the long half-life of Ins(1:2cyc,4,5) P_3 is incompatible with a Ca^{2+} -mobilizing function.

There are now a number of kinetic investigations of 5-phosphatase activities (Table 1). A considerable variation has been obtained, even in repeated studies on the same tissue, such as in platelets and liver (Table 1). Some of this inconsistency predates the commercial availability of Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , and it is possible that the difficulties in preparing pure substrate on a sufficient scale introduced unsuspected contaminants into some preparations. In the more recent studies in liver [40], brain [21], pancreatic islets [18] and skeletal muscle [26] the particle-associated 5-phosphatase has been assigned a K_m for Ins(1,4,5) P_3 in the range 15–19 μ M. However, some other cells, such as erythrocyte ghosts [12,19] and insulin-secreting RINm5F cells [41], may genuinely have a particle-associated 5-phosphatase with slightly lower affinity (K_m 25–30 μ M). The affinity of the enzyme activities in platelets remains inexplicably controversial (Table 1). The K_m of 5-phosphatase activity towards Ins(1,4,5) P_3 is frequently around 10-fold less than that for Ins(1,3,4,5) P_4 (Table 1), although the V_{max} is highest for the Ins(1,4,5) P_3 , again by a factor of around 10 [21,30,40].

There is uncertainty over the relationship between the soluble and membrane-associated forms of the 5-phosphatase. Perhaps in some cell types there may be a single enzyme species that reversibly associates with the membrane, but there may also be isoenzymes. In platelets and brain there are significant kinetic differences between the soluble and particle-bound 5-phosphatase activities (Table 1), which could be due to isoenzymes, but may alternatively be due to unrecognized regulatory factors. The resolution of this issue requires the purification of the particle-bound activity, and some progress in this direction has been made with the solubilization of the enzyme from platelet membranes using 1 M-NaCl [25].

More than one cytosolic form of the enzyme has been described in brain. The 'type 1' soluble enzyme has K_m values for Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 of 3 μ M and

Table 1. Values obtained for the substrate affinity of 5-phosphatase activities

P, particulate; S, soluble. See the text for further details.

Tissue	K_m (μM) for:		
	Ins(1,4,5) P_3	Ins(1,3,4,5) P_4	Ins(1:2cyc,4,5) P_3
Human erythrocyte, P [12]	25	—	—
Human erythrocyte, P [19]	30	—	—
Rat liver, P [40]	15	1.2	—
Rat liver, P [20]	1.4	—	—
Rat liver, S [20]	1.0	—	—
Rat brain, P [19]	50	—	—
Rat brain, P [21]	18	3	—
Rat brain, S, type 1 [21]	3	0.8	—
Rat brain, S, type 2 [21]	18	> 150	—
Human platelet, P [25]	100	—	—
Human platelet, S [25]	40	—	—
Human platelet, S [30]	6	0.8	—
Human platelet, S [56]	17	—	—
Human platelet, S [32]	75	—	1000
Human platelet, S [16]	30	—	—
Rat pancreatic islet, P [18]	16	—	—
Rabbit skeletal muscle, P [26]	15.5–18.7	—	—
Rabbit skeletal muscle, S [26]	24	—	—
RINm5F cells, P [41]	30	—	—
Bovine aorta, S [59]	12	—	—

0.8 μM respectively [21]. The 'type 2' enzyme has lower affinity for Ins(1,4,5) P_3 (K_m 18 μM), and a particularly poor affinity for Ins(1,3,4,5) P_4 (K_m > 150 μM) [21]. The two soluble activities are also different in size: the 'type 1' enzyme is around 60 kDa, and the 'type 2' activity is about 160 kDa. It would be interesting to know if these putative multiple species of 5-phosphatase occur within the same cell types, but this question cannot be answered by studying extracts from a heterogeneous tissue such as brain. It is also worth re-emphasizing that PtdIns-(4,5) P_2 5-phosphatase may attack Ins(1,4,5) P_3 [27,28], thereby contributing to this apparent multiplicity of 5-phosphatase activities.

The kinetic information on partly or even highly purified enzymes cannot accurately predict the fluxes of inositol phosphate metabolites *in vivo*, without more information on intracellular levels of these compounds. It should also be appreciated that some of the K_m values in Table 1 may not be applicable *in vivo*, if physiological factors were to significantly alter the enzyme activities.

For example, it is not known if ATP, which inhibits the 5-phosphatase [21,22,26,53], does so by modifying the V_{max} , or the K_m .

Whilst the soluble 5-phosphatase is by definition an intracellular enzyme, the active site of the particle-bound Ins(1,4,5) P_3 5-phosphatase has also been shown to be intracellular in erythrocytes [42], liver [40] and intestinal epithelial cells [48]. Such observations are relevant to those experiments where the pathways of inositol phosphate metabolism have been analysed by addition of precursors to permeabilized cells and homogenates. In these cases, the substrates are potentially exposed to enzymes on the outer surface of the cell that would not normally attack intracellular inositol phosphates. For example, intestinal cells utilize predominantly extracellular phosphatases to non-specifically remove all three phosphates from Ins(1,4,5) P_3 [48]. Moreover, the possibility that Ins P_5 may act extracellularly [6] may provide an explanation for its metabolism by extracellular phosphatases [149]. Thus it is important to separate intra- and extracellular enzymes of inositol phosphate metabolism.

The particle-bound 5-phosphatase is predominantly associated with plasma membranes [15,17,18,20,22], which has led to speculation that Ins(1,4,5) P_3 is both synthesized and dephosphorylated at the same region of the cell. However, at least in hepatocytes the situation is more complex. Here, the 5-phosphatase is concentrated at the bile canalicular region of the cell, possibly as a consequence of the enzyme being transferred from the sinusoidal region in endosomes [40]. Since Ins(1,4,5) P_3 is presumably generated at the sinusoidal pole, this region of the cell may have a locally high concentration of Ins(1,4,5) P_3 . It is difficult to estimate the extent of any concentration gradient of Ins(1,4,5) P_3 that may occur without more information on intracellular diffusion rates. Possibly the best current estimate of this rate of diffusion may be obtained from the delay that follows the micro-injection of Ins(1,4,5) P_3 into the arhabdomeral lobes of *Limulus* photoreceptors, before Ca^{2+} is released in the rhabdomeral lobes [49,50]. In these experiments, a Ca^{2+} -mobilizing concentration of Ins(1,4,5) P_3 diffused about 50 μm in 3 s. Thus it may take 0.6 s for Ins(1,4,5) P_3 to diffuse the 10 μm separating the sinusoidal and canalicular regions of the hepatocyte. This slight delay may have two important consequences for Ins(1,4,5) P_3 . Firstly, the opportunity for Ins(1,4,5) P_3 to encounter the intracellular Ca^{2+} store will be improved, particularly if the pool is close to the sinusoidal plasma membrane [51,52]. Secondly the hepatic Ins(1,4,5) P_3 3-kinase, being a soluble enzyme (see below), may initially provide the preferred route of Ins(1,4,5) P_3 metabolism.

In receptor-activated cells the levels of Ins(1,4,5) P_3 are regulated by a complex interplay between phospholipase C activity and the 5-phosphatase and 3-kinase activities that metabolize the Ins(1,4,5) P_3 . Control over the rate of Ins(1,4,5) P_3 metabolism, and hence the life-time of its messenger activities, may be brought about by regulation of the phosphatase (and/or the kinase). Phorbol ester treatment of platelets activates 5-phosphatase activity, so protein kinase C has been implicated as a physiological regulator of Ins(1,4,5) P_3 metabolism [30,56,57]. Majerus and colleagues reported that the soluble platelet 5-phosphatase activity migrated on SDS/polyacrylamide gels with the major protein substrate for phosphorylation by protein kinase C [56]. However, other workers [60]

have separated the phosphorylated protein from the 5-phosphatase, indicating that the latter is not the substrate for protein kinase C (this also indicates that the 5-phosphatase prepared in Majerus's laboratory was not homogeneous). Some workers have further questioned the proposed regulation of 5-phosphatase by protein kinase C, since its inhibition by staurosporine did not affect thrombin-mediated increases in cellular InsP_3 levels [58]. Unfortunately, the latter data are questionable, since concentrations of $\text{Ins}(1,4,5)\text{P}_3$ are not accurately assessed by measuring total InsP_3 , 90 % of which may be $\text{Ins}(1,3,4)\text{P}_3$ [36].

Whilst it is unclear how phorbol ester treatment of platelets activates 5-phosphatase [30,56,57], it is becoming apparent this may not be a widespread phenomenon; both particle-associated and soluble species of 5-phosphatase activity were unaffected by phorbol ester treatment of either astrocytoma cells [61], insulin-secreting RINm5F cells [54] or hepatocytes [62]. Down-regulation of protein kinase C by chronic treatment with phorbol esters also did not affect 5-phosphatase activity in an epithelial cell line from liver [63].

Two soluble 5-phosphatase activities, from macrophages [64] and porcine artery smooth muscle [23], are apparently activated by receptor-mediated elevation of cytosolic Ca^{2+} (from 0.2 to 1 μM). In these cases, the mobilized Ca^{2+} would act to reduce further Ca^{2+} release by stimulating $\text{Ins}(1,4,5)\text{P}_3$ metabolism. However, 5-phosphatase was unaffected by Ca^{2+} in several other cells except at unphysiologically high concentrations (K_i 70–100 μM) [16,21,22,41,42,65,66].

Inhibition of 5-phosphatase *in vitro* by 5 mM-AMP or 5 mM-PP_i are unlikely to occur *in vivo* [22]. $\text{Ins}(1,4)\text{P}_2$ may inhibit (K_i 70–100 μM) [12,42]) but there is little information on $\text{Ins}(1,4)\text{P}_2$ levels *in vivo* (see below). The 5-phosphatase from bovine aorta was inhibited by a racemic mixture of DL- $\text{Ins}(1,3,4)\text{P}_3$ with a K_i of 32 μM [59]. It is important to determine the extent to which this inhibition was due to D- $\text{Ins}(1,3,4)\text{P}_3$, since this could physiologically regulate $\text{Ins}(1,4,5)\text{P}_3$ levels. ATP inhibits the 5-phosphatase [21,22,26,53], but there is no evidence that this is due to control by phosphorylation [22], and intracellular concentrations of ATP seem not to vary sufficiently to significantly regulate the enzyme. Spermine at 2 mM inhibits the 5-phosphatase from liver by 50 % [17]. Whilst these concentrations of spermine are at least double those in unstimulated cells [67], there are agonists that can rapidly increase intracellular levels of this polyamine [68], and this might prolong a $\text{Ins}(1,4,5)\text{P}_3$ signal induced by an appropriate stimulus. Inhibition of 5-phosphatase by fructose 1,6-bisphosphate (K_i 0.33 mM [18]) and 2,3-bisphosphoglycerate (K_i around 0.4 mM [12,18]) may also be important; stimulation of pancreatic islets with glucose increases the concentrations of glycolytic intermediates within the range that would significantly inhibit the 5-phosphatase [18].

Recently a number of analogues of $\text{Ins}(1,4,5)\text{P}_3$ have been synthesized [46,59]. Amongst these is the 1,4,5-trisphosphorothioate analogue of $\text{Ins}(1,4,5)\text{P}_3$, which is an efficient Ca^{2+} -mobilizing agonist [46], but is not attacked by the 5-phosphatase [69]. This may be a useful tool for studying Ca^{2+} mobilization independently of $\text{Ins}(1,4,5)\text{P}_3$ metabolism.

$\text{Ins}(1,4,5)\text{P}_3$ 3-kinase

The discovery of $\text{Ins}(1,3,4,5)\text{P}_4$ [29] led quickly to

the detection of $\text{Ins}(1,4,5)\text{P}_3$ kinase activity in cells [55,70,150]. The kinase from brain was initially reported to phosphorylate $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ readily [70], but this is now known not to be the case [30,33,36]. Since $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(4,5)\text{P}_2$ are also relatively poor substrates [71,72], it is clear that the kinase is highly specific for the 1,4,5-trisphosphate configuration. The enzyme is predominantly soluble in all tissues where its intracellular distribution has been studied [37,41,59,70,71,73–75]. Even in turkey erythrocytes, where significant particulate activity was detected, this only amounted to 25 % of total cellular activity [66]. Most reports put the K_m for $\text{Ins}(1,4,5)\text{P}_3$ in the range 0.2–1.5 μM [41,59,71,72,74–77]. The affinity of the 5-phosphatase is some 10-fold lower (see above), but whether or not this leads to $\text{Ins}(1,4,5)\text{P}_3$ being preferentially phosphorylated depends also on the relative V_{max} values for phosphorylation and dephosphorylation, the intracellular concentration of $\text{Ins}(1,4,5)\text{P}_3$, and possibly also the spatial distribution of the 5-phosphatase and 3-kinase within the cell. The kinase from rat heart has a relatively low affinity for $\text{Ins}(1,4,5)\text{P}_3$ (K_m 5.5 μM [78]), which may contribute to the low levels of $\text{Ins}(1,3,4,5)\text{P}_4$ in stimulated myocytes [78,79] compared to some other cells.

The 3-kinase probably plays a key role in regulating $\text{Ins}(1,4,5)\text{P}_3$ metabolism. There is widespread evidence [41,54,62,65,66,72,74–76,81–84] that increases in Ca^{2+} (from 0.2 to 1 μM) stimulate the 3-kinase in many tissues in a calmodulin-dependent manner. Thus the transient receptor-mediated elevation of cytosolic $[\text{Ca}^{2+}]$ can increase the rate of formation of $\text{Ins}(1,3,4,5)\text{P}_4$. This phenomenon may autocatalytically enhance the Ca^{2+} signal, as $\text{Ins}(1,3,4,5)\text{P}_4$ promotes the Ca^{2+} -mobilizing function of $\text{Ins}(1,4,5)\text{P}_3$ [3–5] and inhibits $\text{Ins}(1,4,5)\text{P}_3$ dephosphorylation (see above).

There are some conflicting reports concerning the effects of Ca^{2+} on the kinase in rat brain. Two laboratories [70,71] noted that at concentrations of $\text{Ins}(1,4,5)\text{P}_3$ less than 0.4 μM , changes in Ca^{2+} from 0.1 to 10 μM inhibited kinase activity up to 3-fold. Since Downes and coworkers [71] showed that Ca^{2+} did not affect the kinase when the $\text{Ins}(1,4,5)\text{P}_3$ concentration was 10 μM , it was proposed that Ca^{2+} increased the K_m of the enzyme. This effect on the K_m has been confirmed by Williamson and colleagues [75], but the latter workers also demonstrated that, as in several other tissues [41,75,76,78], Ca^{2+} increased the V_{max} . Thus two groups [75,82] have now shown that when the concentration of $\text{Ins}(1,4,5)\text{P}_3$ exceeds the K_m value, Ca^{2+} /calmodulin activates the kinase from rat brain by 50–100 %. It is perhaps not surprising that these rather complex effects of Ca^{2+} , possibly superimposed upon some subtle aspects of the incubation media or the enzyme preparations, led to conditions where changes in Ca^{2+} concentration did not increase kinase activity [71].

In some cases stimulation of intact cells brings about a persistent and presumably covalent modification that increases $\text{Ins}(1,4,5)\text{P}_3$ kinase activity. This phenomenon was observed in a malignant human T-cell line when antigen receptors were activated, and was mimicked by the stimulation of protein kinase C with phorbol esters [76]. In hepatocytes, $\text{Ins}(1,4,5)\text{P}_3$ kinase was persistently activated by stimulation of both protein kinases A and C together, though neither protein kinase could bring about this effect alone [62]. The $\text{Ins}(1,4,5)\text{P}_3$ kinase in permeabilized RINm5F cells (from an insulinoma) was activated by either phorbol esters or cyclic AMP [54]. Clearly

cells utilize several mechanisms to finely regulate $\text{Ins}(1,4,5)\text{P}_3$ kinase activity, closely controlling the intracellular signalling functions of both substrate and product.

Some determinations of the molecular mass of the kinase have been made: a partially purified preparation from rat brain appeared, on gel filtration, to have a molecular mass of 200 kDa [71]. This value is reasonably close to that of 160 kDa reported by Williamson and coworkers [75] who purified the rat brain enzyme to apparent homogeneity. The native enzyme appears to consist of two monomers of 53 kDa, each of which is bound to calmodulin [75]. The kinase from pig aortic smooth muscle has also been purified to apparent homogeneity, although in this case a molecular mass of 93 kDa was obtained by SDS/polyacrylamide-gel electrophoresis [74].

$\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase

Varying degrees of $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase activity have recently been observed in rat basophil leukaemic (RBL-2H3) cells [53], human erythrocytes [86], brain cytosol [87] and GH3 cells [88]. Clearly this activity could have important consequences for the maintenance of $\text{Ins}(1,4,5)\text{P}_3$ levels and the regulation of cell signalling. However, the physiological activity of the 3-phosphatase is not known, and even its contribution to $\text{Ins}(1,3,4,5)\text{P}_4$ metabolism *in vitro* varies considerably with the incubation conditions. For example, because of the Mg^{2+} -dependence of $\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase, proportionally more 3-phosphatase activity was observed in the absence of Mg^{2+} [86]. In homogenates of GH3 cells [88] and RBL-2H3 cells [53], < 6% of total $\text{Ins}(1,3,4,5)\text{P}_4$ dephosphorylation was due to the 3-phosphatase. In contrast, in a particulate fraction prepared from the RBL-2H3 cells, up to 50% of $\text{Ins}(1,3,4,5)\text{P}_4$ dephosphorylation led to $\text{Ins}(1,4,5)\text{P}_3$ production [53]. Whilst neither a cell homogenate, nor a fraction derived from it, may give a sound indication of the relative rates of $\text{Ins}(1,3,4,5)\text{P}_4$ 3- and 5-phosphatase *in vivo*, these experiments are significant because they were performed with 2 mM-ATP. This reduces 5-phosphatase activity [21,22,53], which by itself may divert more $\text{Ins}(1,3,4,5)\text{P}_4$ through the 3-phosphatase, but perhaps the latter enzyme activity is activated by ATP.

In most of the above studies, it remains to be demonstrated that the 3-phosphatase is located inside the cell, where the $\text{Ins}(1,3,4,5)\text{P}_4$ is produced. The exception appears to be brain, where considerable 3-phosphatase activity was found in some anion-exchange fractions of a 100 000 g supernatant [87]. Again, the activity of this enzyme relative to the 5-phosphatase in the whole cell is unknown. Finally, it should also be appreciated that all these 3-phosphatase assays were performed with trace amounts of substrates. The true significance of this pathway may not become clear until physiologically relevant substrate concentrations are employed.

$\text{Ins}(1,4)\text{P}_2$ 4-phosphatase

Storey *et al.* [15] reported that liver homogenates dephosphorylated $[4\text{-}^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$ to both $[^{32}\text{P}]\text{InsP}$ and $^{32}\text{P}_i$. The $^{32}\text{P}_i$ was believed to have been directly released from the $\text{Ins}(1,4)\text{P}_2$, particularly since indirect evidence indicated that $[^{32}\text{P}]\text{Ins}4\text{P}$ was dephosphorylated at a 100-fold slower rate than $\text{Ins}(1,4)\text{P}_2$. This was an incorrect identification of $\text{Ins}(1,4)\text{P}_2$ 4-phosphatase activity, partly

because the rate of $\text{Ins}4\text{P}$ metabolism was underestimated [89]. Additionally Storey *et al.* [15] used $[4\text{-}^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$ that was prepared by Ca^{2+} activation of phospholipase C in ^{32}P -labelled erythrocyte ghosts [12]. These preparations are now known to include unidentified contaminants that release $^{32}\text{P}_i$ upon incubation with liver homogenates [89]. It may now be worth investigating whether blowfly salivary glands rapidly metabolize $\text{Ins}4\text{P}$, because none was detected during the dephosphorylation of $[4\text{-}^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$, which led to the conclusion that this tissue had a $\text{Ins}(1,4)\text{P}_2$ 4-phosphatase [13].

Another identification of $\text{Ins}(1,4)\text{P}_2$ 4-phosphatase activity seems to be incorrect; Takimoto *et al.* [99] reported that they had purified this enzyme to homogeneity from brain, but Inhorn & Majerus [103] have proposed that Takimoto and coworkers incorrectly identified the products of the reaction, such that the enzyme was in fact the 1-phosphatase. Certainly the Takimoto enzyme [99] shares some of the properties of the 1-phosphatase, including size (42 kDa), and its inhibition by Ca^{2+} and Li^+ (see below).

Although evidence obtained from many cell types now indicates that less than 5% of $\text{Ins}(1,4)\text{P}_2$ dephosphorylation is performed by a 4-phosphatase [89–97], this activity persists in some circumstances. For example, when a rat brain supernatant was incubated with 700 μM - $\text{Ins}(1,4)\text{P}_2$, about 30% of the InsP products were $\text{Ins}1\text{P}$ [98]. A small proportion of 4-phosphatase activity was also detected in homogenates of parotid acinar cells [85] when Li^+ was used to inhibit $\text{Ins}(1,4)\text{P}_2$ 1-phosphatase. Li^+ acts uncompetitively, and therefore more acutely at higher substrate concentrations (see below). Thus, 4-phosphatase was not detected when 0.25 μM - $\text{Ins}(1,4)\text{P}_2$ was added to the parotid homogenates, but at 100 μM - $\text{Ins}(1,4)\text{P}_2$, 30% of the InsP products were $\text{Ins}1\text{P}$.

The experiments with parotid homogenates [85] suggest that certain conditions need to prevail if $\text{Ins}(1,4)\text{P}_2$ 4-phosphatase is to be a physiologically relevant activity *in vivo*. Firstly $\text{Ins}(1,4)\text{P}_2$ levels need to be relatively high, maybe approaching 100 μM , and it is unclear if this is possible. Secondly, the 1-phosphatase activity must be sufficiently inhibited: Li^+ is not a physiologically relevant inhibitor (except when it is used to treat certain types of mania) but more relevant inhibition may be effected by Ca^{2+} [102], ATP [100] or competition by $\text{Ins}(1,3,4)\text{P}_3$ (see below). It also needs to be established that the 4-phosphatase has a specificity for $\text{Ins}(1,4)\text{P}_2$, so that the enzyme is not primarily involved in the metabolism of other substrates *in vivo*. The question of substrate specificity does introduce a further problem that might be worth studying, namely whether this 4-phosphatase is responsible for the dephosphorylation of $\text{Ins}(1:2\text{cyc},4)\text{P}_2$ [32], a suggestion raised tentatively in Fig. 1.

$\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4)\text{P}_3$ 1-phosphatase

The first suggestion that $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4)\text{P}_3$ were hydrolysed by the same enzyme arose from the erroneous suggestion that it was a 4-phosphatase [55]. Later work correctly identified a 1-phosphatase attack on $\text{Ins}(1,3,4)\text{P}_3$ that was inhibited by $\text{Ins}(1,4)\text{P}_2$ [42,102]. That a single enzyme activity was responsible has been proven following purification of the phosphatase [101,102]. This enzyme has been called an 'inositol polyphosphate 1-phosphatase' [102,103] but, as pointed

out by Ragan and colleagues [101], this title gives a misleading impression of the specificity of the enzyme since, for example, it does not attack $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$ [96,98,101].

Inhorn & Majerus [102,103] measured the affinities of the enzyme in brain and several other tissues: the K_m values for $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ respectively were 5–20 μM and 4–5 μM [102,103]. Ragan and coworkers [101] obtained values for the brain enzyme that were substantially above these ranges: 500 μM for $\text{Ins}(1,3,4)\text{P}_3$ and 40 μM for $\text{Ins}(1,4)\text{P}_2$. Other workers have obtained K_m values for $\text{Ins}(1,4)\text{P}_2$ that add to this inconsistency: 18 μM [89], 0.8–0.9 μM [20,90] and 40 μM [25]. Some groups [89,101] made these determinations with racemic mixtures of substrates, rather than D-enantiomers, but this is not the cause of the disagreement (C. I. Ragan, personal communication). Perhaps there are some unsuspected effects due to enzyme preparations or incubation media.

The 1-phosphatase is a largely cytosolic enzyme [25,42,103], although in some tissues up to 20 % of total activity may be particle-bound [42,103]. The cytosolic and membrane-bound enzymes are similar, at least in brain, since antibodies raised to the former will also bind to the latter [103]. It is generally agreed that the molecular mass of the phosphatase is 40–49 kDa [25,89,96,101,103].

In brain, the 1-phosphatase is inhibited about 30 % as $[\text{Ca}^{2+}]$ rises from 0.1 to 1 μM [102]. ATP also inhibits the hepatic enzyme [22,100], but it is not known if this represents control of enzyme activity by phosphorylation. Inhibition of the 1-phosphatase could direct more $\text{Ins}(1,3,4)\text{P}_3$ through the $\text{Ins}(1,3,4)\text{P}_3$ kinase and $\text{Ins}(1,3,4)\text{P}_3$ 4-phosphatase, and possibly more $\text{Ins}(1,4)\text{P}_2$ through a 4-phosphatase.

Shears and colleagues [24,42] reported that the 1-phosphatase was insensitive to 50 mM- Li^+ . In contrast, many other workers [55,91,96–98,101,102] showed that as little as 3 mM- Li^+ was a potent inhibitor. This controversy has been explained [89,91,102] by demonstrating that Li^+ is primarily an uncompetitive inhibitor, which therefore acts less dramatically with decreasing substrate concentration and almost undetectably so in the nanomolar range used in Shears' original experiments. It is therefore surprising that some workers [104] observed that 3 mM- Li^+ inhibited, by 50 %, the dephosphorylation of 2 nM- $\text{Ins}(1,3,4)\text{P}_3$ by a partially purified phosphatase from rat brain. Perhaps there may also be a tissue-dependent non-competitive aspect to the inhibition by Li^+ [89].

$\text{Ins}(1,3,4)\text{P}_3$ 4-phosphatase and $\text{Ins}(3,4)\text{P}_2$ 4-phosphatase

Various groups have demonstrated that $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(3,4)\text{P}_2$ can both be hydrolysed by a 4-phosphatase [88,93,100,103,105], but it has not yet been shown if these reactions are catalysed by a single enzyme. These phosphatase activities may be Li^+ -insensitive [105], although it is difficult to be sure as trace quantities of substrate were used, and Li^+ may act uncompetitively. In brain both activities are Mg^{2+} -independent, which is unusual amongst the enzymes of inositol phosphatase metabolism, with the exception of the $\text{Ins}(1,3)\text{P}_2$ 3-phosphatase. Inhorn & Majerus [103] exploited the Mg^{2+} -independence of the $\text{Ins}(1,3,4)\text{P}_3$ 4-phosphatase to determine the contribution this enzyme made to $\text{Ins}(1,3,4)\text{P}_3$ metabolism, compared to the Mg^{2+} -dependent 1-phosphatase. $\text{Ins}(1,3,4)\text{P}_3$ dephosphorylation in homogenates of a number of tissues was assayed, in the presence

and absence of Mg^{2+} , and in brain the 4-phosphatase accounted for 72 % of the total. In every other tissue analysed, the 4-phosphatase was a minor pathway (< 20 % of total).

$\text{Ins}(1,3)\text{P}_2$ 3-phosphatase

The two possible InsP products of $\text{Ins}(1,3)\text{P}_2$ metabolism are stereoisomers unresolved by non-chiral h.p.l.c. techniques, but the 3-phosphatase attack upon $\text{Ins}(1,3)\text{P}_2$ has been characterized by using ^{32}P -labelled substrates [105]. The enzyme is Mg^{2+} -independent [105]. ATP inhibits the phosphatase [100], but it is not known if this is due to control by enzyme phosphorylation; it may be a response that is typical of several inositol phosphate phosphatases.

$\text{Ins}(1:2\text{cyc})\text{P}$ 2-phosphohydrolase

Dawson & Clarke [106] identified this enzyme in 1972. $\text{Ins}(1:2\text{cyc})\text{P}$ may be formed *in vivo* either by sequential removal of the 5- and then the 4- phosphates from $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ [32], or by phospholipase C-catalysed hydrolysis of PtdIns [107]. $\text{Ins}(1:2\text{cyc},4)\text{P}_2$ and $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ are apparently not substrates of the hydrolase [108]. The enzyme has been purified to apparent homogeneity from human placenta; it is a 55 kDa dimer that has a $K_m > 50 \mu\text{M}$ for $\text{Ins}(1:2\text{cyc})\text{P}$ [108].

The hydrolase is inhibited *in vitro* when either Ins or $\text{Ins}1\text{P}$ exceeds 100 μM [108], so these compounds could contribute to the accumulation of $\text{Ins}(1:2\text{cyc})\text{P}$ observed in some stimulated cells [107,110]. A much more potent inhibitor of the hydrolase is $\text{Ins}2\text{P}$ (K_i 4 μM [108]). There have been occasions when an InsP in a cell extract has been reported to be $\text{Ins}2\text{P}$ [37,92,111] but usually this InsP has not been rigorously analysed, and could have been mistaken for other monophosphates (e.g. see [112]). Additionally, $\text{Ins}2\text{P}$ can be produced quite artificially *in vitro* by acid-based cell extraction techniques hydrolysing $\text{Ins}(1:2\text{cyc})\text{P}$ to $\text{Ins}1\text{P}$ and $\text{Ins}2\text{P}$ [107]. However, Sherman and colleagues [109] have recently obtained more convincing evidence that there is around 10 μM - $\text{Ins}2\text{P}$ in neutral extracts of rat cerebral cortex, and this would be likely to inhibit the hydrolase.

InsP phosphatase

It has been known for over 20 years that mammalian cells contain a soluble phosphatase activity towards $\text{Ins}1\text{P}$ and $\text{Ins}3\text{P}$ [113], so several groups have purified the enzyme to varying degrees. Most determinations of its native molecular mass range from 46 to 60 kDa [114–118], and it appears to consist of two monomers each of 29 kDa [114,116]. However, there may be conditions that cause the subunits of the enzyme to aggregate into a polymer of 210 kDa [119]. This phosphatase is remarkably heat-stable [113], a factor which has been exploited to facilitate its purification [118]. The proposal [98] that a single enzyme dephosphorylates $\text{Ins}1\text{P}$, $\text{Ins}3\text{P}$, $\text{Ins}4\text{P}$ and $\text{Ins}6\text{P}$ has now been proven in an enzyme from brain purified to homogeneity [114]. The same enzyme probably also attacks $\text{Ins}5\text{P}$ [98]. The phosphatase has similar affinities for all of its substrates (K_m 0.1–0.2 mM [98,114–118]), although the V_{max} for the 4,6 enantiomeric pair may be 3-fold greater than for the 1,3 pair [114]. $\text{Ins}(1:2\text{cyc})\text{P}$ and $\text{Ins}2\text{P}$ are usually the only inositol monophosphates that are not attacked by the phosphatase [98,113]. In fact $\text{Ins}2\text{P}$ will inhibit the enzyme, although the K_i is 0.3 mM [115,119], and it is

doubtful if these concentrations occur *in vivo*. Similar concentrations of various isomers of InsP_2 , InsP_3 , InsP_4 and InsP_5 did not inhibit the phosphatase [98]. Interestingly, the monophosphatase in avian erythrocytes seems to be atypical, in both its ability to hydrolyse Ins2P , and its almost total inhibition by 0.5 mM-InsP_5 [117].

Note that Ins3P is not only produced by dephosphorylation of Ins(3,4)P_2 (Fig. 1), but is also formed by cyclization of glucose 6-phosphate [113]. As the InsP phosphatase catalyses a common step in two pathways of cellular Ins supply, inhibition of this enzyme *in vivo* by Li^+ [114–120] can dramatically reduce the supply of precursors for inositol lipid resynthesis, particularly in those tissues such as brain that have limited access to extracellular Ins [120]. This in turn, so it is argued [120,121], will be particularly effective in depleting levels of inositol lipids in those cells that are vigorously activated, as may be the case in some forms of mania. The successful treatment of this illness with Li^+ may therefore be in no small part due to attenuation of receptor-mediated inositol lipid turnover, specifically in the overactive cells.

Current knowledge of inositol phosphate metabolism (Fig. 1) does not account for the supply of all potential substrates for the monophosphatase *in vivo*: Ins5P may be present in GH3 cells [112], and also rat cerebral cortex [98,120]. The latter may also contain Ins2P [109]; their metabolic origins are unknown. Actually, the enzyme is not specific for inositol phosphates; other substrates are AMP, GMP, and β -glycerophosphate [113–116].

Ins(1,3,4)P_3 6-kinase

This reaction was first described by two laboratories working independently: an InsP_4 was found to be formed in an ATP-dependent manner when Ins(1,3,4)P_3 was added to either liver homogenates [22] or permeabilized adrenal glomerulosa cells [122]. The latter study also indicated that this InsP_4 was present in intact adrenal cells. The InsP_4 formed by the cell extracts was cautiously identified as Ins(1,3,4,6)P_4 [22,123]. More elegant and extensive work has confirmed the presence of Ins(1,3,4,6)P_4 in other tissues [133]. Receptor-initiated changes in the cellular concentration of Ins(1,3,4,6)P_4 appear to be relatively minor, even when levels of Ins(1,3,4)P_3 increase dramatically. For example, in macrophages stimulated with platelet-activating factor, Ins(1,3,4)P_3 levels increased 60-fold within 40 s and then decreased over the following 10 min; during that time, Ins(1,3,4,6)P_4 levels only rose 5-fold [133]. In stimulated hepatocytes [125] and adrenal glomerulosa cells [95], even when Li^+ was used to inhibit Ins(1,3,4)P_3 dephosphorylation, the accumulation of the trisphosphate was several-fold greater than that of Ins(1,3,4,6)P_4 . So, relative to the rate of Ins(1,3,4)P_3 dephosphorylation, the 6-kinase has a limited activity, despite its relatively high affinity for Ins(1,3,4)P_3 , at least in liver (K_m $0.3 \mu\text{M}$ [125]).

Neither Ins(1,4)P_2 , Ins(1,3)P_2 nor Ins(3,4)P_2 are phosphorylated by the 6-kinase [133,100]. The enzyme appears to be largely soluble, with an apparent molecular mass of 30 kDa [133]. Kinase activity was unaffected by either cyclic AMP or by receptor-mediated changes to Ca^{2+} [125,133].

Ins(1,3,4,6)P_4 5-kinase

Ins(1,3,4,6)P_4 can be further phosphorylated to InsP_5 in

extracts from brain [133], adrenal glomerulosa cells [124], and liver (S. B. Shears, C. J. Kirk & R. H. Michell, unpublished work). Only in brain has the structure of the InsP_5 been shown to be Ins(1,3,4,5,6)P_5 [133]. Ins(1,3,4,6)P_4 5-kinase from brain is a soluble enzyme which has a molecular mass of around 30 kDa [133]. This enzyme extends the pathway of receptor-activated inositol phosphate metabolism to include InsP_5 (Fig. 1), and may contribute to a relatively small agonist-induced stimulation of InsP_5 levels [94,133]. However, such increases have not always been observed [126] and intriguingly, a human epidermoid carcinoma cell line responds to stimulation with a transient decrease in InsP_5 levels [127]. A complication in these studies is that Ins(1,3,4,5,6)P_5 is also produced by phosphorylation of Ins(3,4,5,6)P_4 by a soluble 1-kinase [128] (see below). The relative contributions of the two kinases to InsP_5 synthesis *in vivo* are unclear, but key roles for these enzymes might be predicted from recent evidence that InsP_5 is an extracellular signal [6].

OTHER METABOLIC STEPS?

Several factors indicate that the information in Fig. 1 is incomplete. For example, Ins(1,3,4,6)P_2 can be dephosphorylated, but the products are unknown [133]. Secondly, the metabolic fate of Ins(1,3,4,5,6)P_5 is not yet clear. Thirdly, Ins(4,5)P_2 has been tentatively identified in small quantities in GH3 cells [92,112] and may be produced by the action of Ins(1,4,5)P_3 1-phosphatase [112], possibly similar to that recently observed in homogenates of *Dictyostelium* [130]. The intracellular origins of Ins2P and Ins5P are also unknown. Another metabolite, MeIns(1,4,5)P_3 , has been identified in cells quenched and extracted with media that contain methanol [129], but this may turn out to be the result of non-enzymic methanolysis of PtdIns(4,5)P_2 [39].

Inositol phosphates may be interconverted by a further series of reactions [9], and one of the intermediates may be Ins(3,4,5,6)P_4 [131,132]. In an effort to identify the source of this compound, Stephens and coworkers (L. R. Stephens, P. T. Hawkins & C. P. Downes, unpublished work) performed short-term labelling of chick erythrocytes with ^{32}P and found that the 5-phosphate in the Ins(3,4,5,6)P_4 had a higher specific activity than the other individual phosphates. These data suggest that the precursor of this InsP_4 is Ins(3,4,6)P_3 . It is already clear that these metabolites have at least one link with those inositol phosphates which originate from receptor activation, since phosphorylation of Ins(3,4,5,6)P_4 and Ins(1,3,4,6)P_4 produce the same InsP_5 [128,133]. These reactions are a still further complication in the study of inositol phosphate metabolism.

INTRACELLULAR CONCENTRATIONS OF INOSITOL PHOSPHATES

The introduction outlines the difficulties in interpreting data on inositol phosphate fluxes *in vivo* from many of the earlier radiolabelling experiments. In addition, these experiments tell us little about the chemical concentrations of the intermediates because their specific radioactivities are usually not known, and cannot be assumed to be equivalent unless equilibrium labelling is demonstrated (see below). Nevertheless, knowledge of these intracellular concentrations is important, firstly so as to determine the physiological significance of the

effects of these compounds obtained with *in vitro* systems. Secondly, it facilitates the understanding of the control of metabolic fluxes of inositol phosphates *in vivo*. The studies of this problem that are considered below will primarily be those where the isomers of the inositol phosphates have been distinguished, since mass determinations of bulk InsP , InsP_2 or InsP_3 (e.g. [135]) do not provide enough useful information.

A wide variety of cell types utilize the inositol lipid signalling system to respond to various agonists. Therefore it would not be expected that, in all cells, a particular inositol phosphate would attain the same basal and stimulated concentrations. However, it is arguable that resting and stimulated concentrations of $\text{Ins}(1,4,5)\text{P}_3$ would be respectively below and above the K_m for Ca^{2+} release. Since the latter parameter is around $0.8\ \mu\text{M}$ in many cell types [1], this sets some expectations of the range of $\text{Ins}(1,4,5)\text{P}_3$ levels in cells. Even this prediction has the drawback that it ignores the paradoxical disagreement between the K_m for Ca^{2+} release and the K_d for $\text{Ins}(1,4,5)\text{P}_3$ binding to its receptor ($< 80\ \text{nm}$, e.g. [136]).

Some estimates of intracellular concentrations of inositol phosphates have been based on attempts at labelling these compounds to isotopic equilibrium with radioactive inositol. This procedure can take several days [137] possibly in part because there are metabolically distinct pools of inositol lipids [138,139], so it is better suited to cells in culture rather than isolated cell preparations with short-term viability.

Cultured anterior pituitary cells were labelled for 3 days with both $[^{32}\text{P}]\text{P}_i$ and $[^3\text{H}]\text{inositol}$ [94], and the inositol phosphates were extracted from the cell and separated by h.p.l.c. The specific activity of $[^{32}\text{P}]\text{ATP}$ was evaluated, and was assumed to be identical to the specific activity of ^{32}P in $\text{PtdIns}(4,5)\text{P}_2$. The $^{32}\text{P}/^3\text{H}$ ratio of the inositol lipids was used to calculate the specific activity of inositol, and hence the mass of the inositol phosphates. The $\text{Ins}(1,4,5)\text{P}_3$ levels were estimated to be $56\ \text{nm}$ after 30 min stimulation with gonadotrophin-releasing hormone, which was about a 2.5-fold increase above basal values. These concentrations of $\text{Ins}(1,4,5)\text{P}_3$ are substantially below estimates from other cells (see below), so it is possible that some of the assumptions in the rather involved procedures led to an underestimation of inositol phosphate mass.

Other workers [80,137] have incubated cells with $[^3\text{H}]\text{inositol}$ and assessed the attainment of equilibrium labelling by empirically confirming that inositol and inositol lipids have the same specific activity. Then individual inositol phosphates were separated by h.p.l.c., and their concentrations were determined from the amount of ^3H therein. Michell *et al.* [137] estimated that in HL60 cells $\text{Ins}(1,4,5)\text{P}_3$ levels rose from a basal level of $0.4\ \mu\text{M}$ to $1.6\ \mu\text{M}$ within 35 s of stimulation with fMet-Leu-Phe, which is within the range expected for effecting Ca^{2+} mobilization. The $\text{Ins}(1,3,4)\text{P}_3$ levels were measured simultaneously, and these increased from a basal level of $1.1\ \mu\text{M}$ to $3.6\ \mu\text{M}$ upon stimulation [137].

Similar experiments with AR42J pancreatoma cells [80] indicated that the resting $\text{Ins}(1,4,5)\text{P}_3$ concentration was $2\ \mu\text{M}$, increasing to $25\ \mu\text{M}$ after stimulation with substance P. These basal levels of $\text{Ins}(1,4,5)\text{P}_3$ are above the K_m for Ca^{2+} mobilization (see above), and may contribute to the relatively high concentration of cytosolic Ca^{2+} in unstimulated AR42J cells. However, the authors of this work recognized that some of the putative

$\text{Ins}(1,4,5)\text{P}_3$ may have been compartmentalized, or contaminated with an unknown inositol phosphate. There are several potential sources of an unsuspected inositol phosphate, since the metabolism of these compounds is still incompletely understood.

Some work with a human T-lymphocyte cell line [126] suggests an additional problem for equilibrium labelling experiments. The longer that these cells were labelled with $[^3\text{H}]\text{inositol}$, the greater the amount of radioactivity that was incorporated into InsP_3 , even when the specific activities of inositol lipids was no longer increasing [126]. This may have been a consequence of a relatively slow accumulation of label into InsP_3 and its precursors, some of which are unknown and may be produced independently of receptor activation with indeterminate specific activities. It seems that equilibrium labelling experiments, which already require the far from trivial task of isolating individual inositol phosphates, should be accompanied by structural determinations of the compound under study to check for purity.

In platelets $\text{Ins}(1,4,5)\text{P}_3$ has been measured by phosphorylating it with $[^{32}\text{P}]\text{ATP}$ using the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase [36]. Basal levels of $0.1\text{--}0.2\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ were elevated to around $1\ \mu\text{M}$ upon maximal stimulation with thrombin, and this change is entirely consistent with the Ca^{2+} -mobilizing properties of this compound. The studies with platelets [36] were particularly informative, as the concentrations of $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ were also measured. Although $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ is not phosphorylated by the 3-kinase (see above), it is converted by acid treatment to non-cyclic $\text{Ins}(1,4,5)\text{P}_3$ [plus a little $\text{Ins}(2,4,5)\text{P}_3$]. By performing assays with the 3-kinase before and after acid treatment, $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ concentration was found to reach a peak at around $1\ \mu\text{M}$ after 30 s treatment with thrombin. Total InsP_3 was measured by gas chromatography and the concentration of $\text{Ins}(1,3,4)\text{P}_3$ was assumed to be that remaining after subtraction of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$. The basal concentration of $\text{Ins}(1,3,4)\text{P}_3$ was around $4\ \mu\text{M}$, rising to $20\ \mu\text{M}$ after 10–30 s stimulation with thrombin.

$\text{Ins}(1,4,5)\text{P}_3$ can now be assayed more simply with specific binding proteins. These have been applied to rabbit neutrophils [140], and hepatocytes [141] and in both cases basal levels of $0.1\text{--}0.2\ \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ were elevated to between 1 and $2.5\ \mu\text{M}$ upon maximal stimulation with agonists. The commercial availability of this binding protein will presumably lead to its widespread use as an assay tool. One problem that remains to be resolved, however, is that the measured receptor-activated increases in $\text{Ins}(1,4,5)\text{P}_3$ levels in brain slices were different when measured with binding proteins, compared with determinations by labelling with $[^3\text{H}]\text{inositol}$ [142].

The apparent signalling activity of $\text{Ins}(1,3,4,5)\text{P}_4$ [3–5] makes it important to determine its intracellular concentration. Unfortunately, there is a shortage of information on this issue, and only a few recent studies on radiolabelled cells have distinguished the isomers of InsP_4 . In these particular cases (e.g. [95,125,133]) levels of $\text{Ins}(1,3,4,5)\text{P}_4$ were less than peak concentrations of $\text{Ins}(1,4,5)\text{P}_3$ which many studies put below $3\ \mu\text{M}$ (see above). There is also very little information on the amounts of InsP and InsP_2 isomers in cells.

N.m.r. revealed that $5\text{--}15\ \mu\text{M}$ InsP_6 was present in heart, kidney, liver and spleen [143]. Similar quantities of InsP_5 were found, which could be the 1,3,4,5,6 isomer

[133], though it remains possible that there is more than one InsP_5 isomer in cells.

In conclusion, there are only a limited number of studies where any progress has been made in determining the intracellular concentrations of inositol phosphates. Even those estimates that do exist may have to be reassessed if it transpires that intracellular compartmentation or binding of inositol phosphates is a significant phenomenon.

PROBLEMS FOR THE FUTURE

Hopefully it is apparent from the above discussion that, in order to fully understand inositol phosphate metabolism in receptor-activated cells, much work remains to be done on the measurement of intracellular concentrations of inositol phosphates, and on the purification of the enzymes and the resolution of their kinetic parameters. The complexities of this metabolic pathway, and the probability that several intermediates fulfil important physiological functions, some perhaps still to be discovered, surely indicates that more information will accumulate on the cellular regulation of inositol phosphate metabolism.

A topic of considerable debate is the contribution to inositol phosphate metabolism made by receptor-activated hydrolysis of PtdIns and $\text{PtdIns}4\text{P}$, and the recent identification of $\text{PtdIns}3\text{P}$ [7] and PtdInsP_3 [8] makes this problem more complex. A number of groups, in work unfortunately beyond the scope of this review, have studied phospholipase C specificity in isolated membranes and permeabilized cells. A problem with such preparations is that they do not always contain inositol lipids in the same proportions that occur in intact cells [85,144,145]. It is therefore desirable to study phospholipase C specificity in intact cells, but it is difficult to distinguish the immediate products of lipid hydrolysis from the inositol phosphates secondarily produced from $\text{Ins}(1,4,5)\text{P}_3$. Perhaps in the future we shall be able to separate these two alternatives with some specific inhibitors of inositol phosphate metabolism that can enter intact cells. The therapeutic potential of such inhibitors will surely be sufficient incentive for their development.

It is beginning to be recognized that the intracellular distribution of the inositol phosphate phosphatases and kinases may be important for the metabolism and physiological functions of the intermediates. For example, in skeletal muscle, there may be a concentration of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity in the T tubules and terminal cisternae of the sarcoplasmic reticulum [26]. This proposed location of the phosphatase was believed to support the hypothesis that inositol lipid turnover mediates pharmacomechanical coupling, since the $\text{Ins}(1,4,5)\text{P}_3$ signal could be deactivated immediately following Ca^{2+} release from the sarcoplasmic reticulum. Signal deactivation is perhaps a slower process in liver cells, in which most of the 5-phosphatase activity may be concentrated away from the region of the cell where Ca^{2+} mobilization is initiated [40]. Currently we have only relatively crude methods for probing the spatial arrangement of the inositol lipid signalling system in cells, and this is an area for future development.

Investigation of the function and metabolism of InsP_5 promises to be an exciting field. For instance, if InsP_5 (and InsP_6) are extracellular signals [6], it is important to discover how they are delivered to the extracellular

environment. Will it transpire that a family of extracellular phosphatases are present to deactivate these compounds? It is intriguing that there are two pathways of InsP_5 synthesis, and it would be interesting to know if there is any interaction between them.

A further puzzle surrounding $\text{Ins}(1,3,4,5,6)\text{P}_5$ is its ability to mimic the apparent Ca^{2+} -mobilizing functions of $\text{Ins}(1,3,4,5)\text{P}_4$ in sea urchin eggs [146]. The authors of this study pointed out that, as some cells may contain relatively high resting levels of InsP_5 , it may be restricted to a subcellular compartment in order to prevent this compound constitutively activating the process controlled by $\text{Ins}(1,3,4,5)\text{P}_4$. The latter view is supported by the predicted limited solubility of InsP_5 in cytosol [44,147]. Since current evidence from several cell types indicates that $\text{Ins}(1,3,4,5,6)\text{P}_5$ synthesis is performed in the cytosol, it may be that this inositol phosphate is subsequently accumulated by a subcellular compartment, or becomes tightly bound to proteins, such as aldolase [148].

There is much potential for the molecular biologists to make an impact on this field. For example, the purification and sequencing of the phosphatases and kinases will help determine the molecular basis for their action and regulation. This work will also help determine the relationships between the various different enzymes, and the extent to which there are isoenzymes.

Finally there is always the unpredictable; only a few years ago who could have guessed some of the recent developments? We can be certain of an exciting future.

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