

## THE ROLE OF PHOSPHOINOSITIDE METABOLISM IN SIGNAL TRANSDUCTION IN SECRETORY CELLS

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### Summary

Activation of a variety of cell surface receptors results in a biphasic increase in the cytoplasmic  $\text{Ca}^{2+}$  concentration, due to the release, or mobilization, of intracellular  $\text{Ca}^{2+}$  stores and to the entry of  $\text{Ca}^{2+}$  from the extracellular space. Stimulation of these same receptors also results in the phospholipase-C-catalysed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with the concomitant formation of inositol 1,4,5-trisphosphate [ $\text{Ins}(1,4,5)\text{P}_3$ ] and diacylglycerol. Analogous to the adenylyl cyclase signalling system, receptor-mediated stimulation of phospholipase C also appears to occur through one or more intermediary guanine nucleotide-dependent regulatory proteins. It is well established that phosphatidylinositol 4,5-bisphosphate hydrolysis is responsible for the changes in  $\text{Ca}^{2+}$  homeostasis. There is strong evidence that  $\text{Ins}(1,4,5)\text{P}_3$  stimulates  $\text{Ca}^{2+}$  release from intracellular stores. The  $\text{Ca}^{2+}$ -releasing actions of  $\text{Ins}(1,4,5)\text{P}_3$  are terminated by its metabolism through two distinct pathways.  $\text{Ins}(1,4,5)\text{P}_3$  is dephosphorylated by a 5-phosphatase to  $\text{Ins}(1,4)\text{P}_2$ ; alternatively,  $\text{Ins}(1,4,5)\text{P}_3$  can also be phosphorylated to  $\text{Ins}(1,3,4,5)\text{P}_4$  by a 3-kinase. Whereas the mechanism of  $\text{Ca}^{2+}$  mobilization is understood, the precise mechanisms involved in  $\text{Ca}^{2+}$  entry are not known; a recent proposal that  $\text{Ins}(1,4,5)\text{P}_3$ , by emptying an intracellular  $\text{Ca}^{2+}$  pool, secondarily elicits  $\text{Ca}^{2+}$  entry will be considered. This review summarizes our current understanding of the mechanisms by which inositol phosphates regulate cytoplasmic  $\text{Ca}^{2+}$  concentrations.

### Introduction

Over 30 years have now passed since the original report by Hokin & Hokin of receptor-stimulated turnover of inositol lipids (Hokin & Hokin, 1953). Today, the impact of this phenomenon on a multitude of important biological systems is widely appreciated. Of significance to the theme of this review is not only the central role of the inositides in secretory mechanisms, but also the major role model secretory systems have played in uncovering the meaning and significance

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of Hokin & Hokin's 'phospholipid effect'. Thus, we note that this phenomenon was originally discovered in studies of a model secretory system, the exocrine pancreas, and that the experimental evidence disclosing its physiological significance (discussed below) was also obtained in this same tissue, although almost 30 years later and some 4000 miles away (Streb, Irvine, Berridge & Schulz, 1983). Since the first papers by the Hokin, the phosphoinositides have enjoyed periods of interest, neglect, rekindled interest, controversy and finally acceptance as important intermediaries in biological signalling processes in a wide variety of systems. The pivotal contributions which resulted in this tumultuous history came from a number of different laboratories. Bob Michell's (Michell, 1975) hypothesis that the phosphoinositides somehow served to couple receptors to cellular calcium mobilization provoked considerable research and criticism. However, further progress in understanding the exact role of inositol lipid turnover in receptor mechanisms was hindered by an imprecise knowledge of the biochemical pathways involved. Only in the early 1980s did the experimental evidence begin to indicate that the initial reaction in stimulated phosphoinositide turnover was the breakdown not of the major known inositide, phosphatidylinositol, but rather a minor phosphorylated derivative, phosphatidylinositol 4,5-bisphosphate (Abdel-Latif, Akhtar & Hawthorne, 1977; Kirk, Creba, Downes & Michell, 1981). Berridge (1983) realized that the water-soluble product of this reaction, inositol 1,4,5-trisphosphate [ $\text{Ins}(1,4,5)\text{P}_3$ ], was a likely candidate for a second messenger to activate the release of  $\text{Ca}^{2+}$  from intracellular stores. Shortly thereafter, he and Robin Irvine, in collaboration with Irene Schulz and Hans-Peter Streb in Frankfurt, demonstrated that this molecule in fact had the predicted biological activity:  $\text{Ins}(1,4,5)\text{P}_3$  in micromolar concentrations rapidly released  $\text{Ca}^{2+}$  from a non-mitochondrial store in permeabilized pancreatic acinar cells (Streb *et al.* 1983). This result was quickly confirmed in a number of different laboratories (Berridge, 1986). It now seems very likely that  $\text{Ins}(1,4,5)\text{P}_3$  is indeed the second messenger signalling the release of intracellular  $\text{Ca}^{2+}$  that occurs on activation of  $\text{Ca}^{2+}$ -mobilizing receptors.

A parallel story evolved from the work in Nishizuka's laboratory (Nishizuka, 1983, 1984*a,b*), which demonstrated that the other product of inositol lipid breakdown, diacylglycerol, was also a cellular messenger; this apparently innocuous intermediate of phospholipid metabolism was shown to be a potent and specific activator of a ubiquitous protein kinase which Nishizuka designated as C-kinase. Again, a model secretory system, the platelet, proved ideal in uncovering the role of this important enzyme in signal transduction.

This article summarizes recent work from a number of laboratories which addresses fundamental questions about this important biological signalling system, with emphasis on the inositol phosphates and their relationship to cellular  $\text{Ca}^{2+}$  metabolism. These questions include the nature of the coupling of receptors to phospholipase C, the metabolism of the inositol phosphates, and the mechanisms or mechanisms by which the inositol phosphates regulate cellular calcium metabolism.

### Receptor control of phospholipase C

The first receptor–effector system for which a role of guanine nucleotides was recognized was the adenylate cyclase system (Rodbell, 1980; Smigel *et al.* 1984). It is now recognized that receptor coupling to adenylate cyclase involves an intermediate guanine nucleotide-dependent regulatory protein (G-protein) designated  $G_s$  (s for stimulation of cyclase). In addition, a second G-protein,  $G_i$ , is also known to mediate the actions of receptors which inhibit adenylate cyclase. Other G-proteins have been described, including transducin, a G-protein which mediates the coupling of photon excitation to the activation of a cyclic GMP phosphodiesterase, and  $G_o$  (o for other), purified from brain, whose function is not yet clearly understood. All these proteins are believed to exist naturally as heterotrimers, composed of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. Receptor interaction changes the conformation of the G-protein such that GTP displaces bound GDP from the  $\alpha$  subunit and dissociates from the  $\beta/\gamma$  complex. The dissociated GTP-bound  $\alpha$  subunit of  $G_s$  activates adenylate cyclase (Fig. 1). However, direct inhibition of

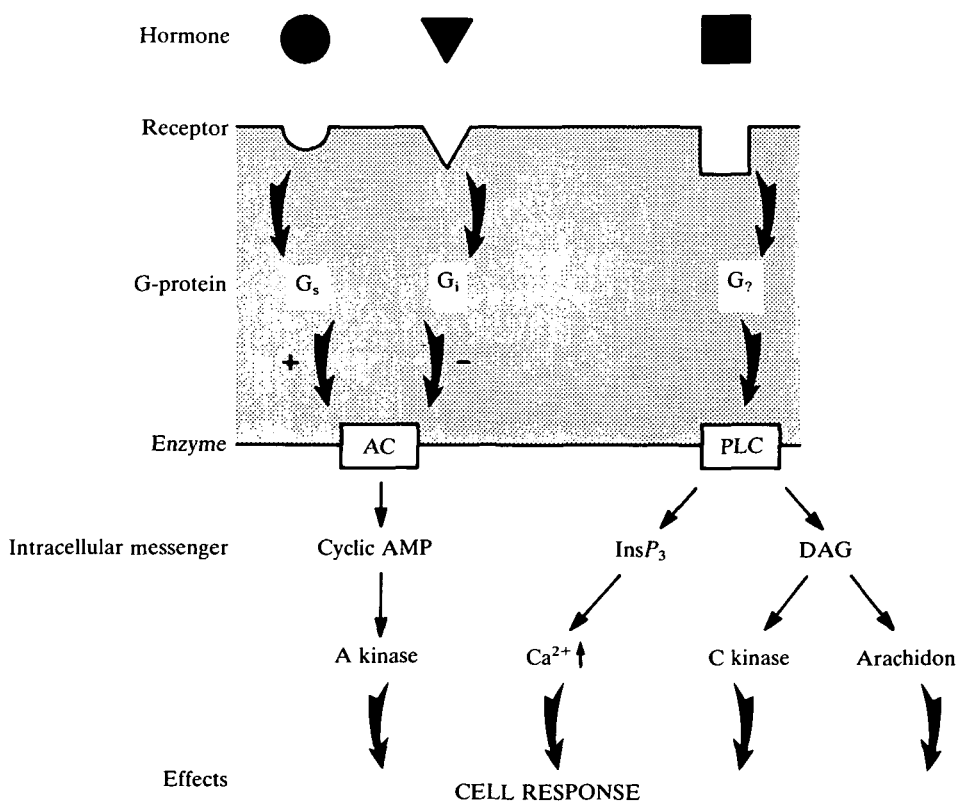


Fig. 1. G-proteins and signal transduction. Known G-proteins ( $G_s$  and  $G_i$ ) regulate the adenylate cyclase system, and there is strong circumstantial evidence that a similar mechanism regulates the polyphosphoinositide phospholipase C (PLC). AC, adenylate cyclase; DAG, diacylglycerol;  $InsP_3$ , inositol trisphosphate.

adenylate cyclase by the  $\alpha$  subunit has not been demonstrated. It has been suggested that inhibition of adenylate cyclase occurs through the liberation of a stoichiometric excess of  $\beta/\gamma$  subunits which associate with the free  $\alpha$  subunits of  $G_s$ , resulting in inhibition (Smigel *et al.* 1984). Nevertheless, it is apparent for the case of the receptors that regulate adenylate cyclase that the dissociation of the G-protein into separate  $\alpha$  and  $\beta/\gamma$  subunits is a necessary prerequisite for accomplishing the necessary effector function. Because receptor activation increases the affinity of the G-proteins for GTP, agonists and GTP generally produce synergistic effects in activating or inhibiting adenylate cyclase. This synergism is especially apparent when non-hydrolysable derivatives of GTP are used. This is because the reassociation of G-protein subunits is normally preceded by the hydrolysis of the bound GTP to GDP. In fact, these non-hydrolysable derivatives of GTP [i.e. GTP- $\gamma$ -S, guanylyl-5'-imidodiphosphate (GppNHp)] bind to G-proteins quite well in the absence of agonists; they can thus produce subunit dissociation and G-protein activation in the absence of receptor activation.

A characteristic of receptors that couple to their effectors through G-proteins is that, in membrane preparations, the binding of agonists (but not antagonists) is inhibited by GTP and non-hydrolysable guanine nucleotides through a decrease in apparent agonist affinity. The earliest indication that receptor coupling to phospholipase C might involve a G-protein mechanism similar to the adenylate cyclase system was the demonstration by a number of investigators that guanine nucleotides decrease the apparent affinity of agonists for receptors of this type (El-Refai, Blackmore & Exton, 1979; Goodhardt, Ferry, Geynet & Hanoune, 1982; Snavely & Insel, 1982; Evans, Martin, Hughes & Harden, 1985). In electrically permeabilized platelets, the effects of thrombin on secretion were potentiated by guanine nucleotides (Haslam & Davidson, 1984*a,b*). More recently, several investigators have demonstrated an activation by guanine nucleotides of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) $P_2$ ] phospholipase C in membranes or in permeable cells (Cockcroft & Gomperts, 1985; Litosch & Fain, 1985; Litosch, Wallis & Fain, 1985; Gonzales & Crews, 1985; Lucas, Bujjalieh, Kowalchuk & Martin, 1985; Uhing, Jiang, Prpic & Exton, 1985; Uhing, Prpic, Jiang & Exton, 1986; Merritt, Taylor, Rubin & Putney, 1986; Taylor, Merritt, Putney & Rubin, 1986; Smith, Cox & Snyderman, 1986). In many cases, the appropriate synergistic interactions between agonists and guanine nucleotides have been reported. Similarly, activation of phospholipase C shows the same relative sensitivity to guanine nucleotide analogues as for regulation of adenylate cyclase: GTP- $\gamma$ -S > GppNHp > GTP (Merritt *et al.* 1986).

An example of guanine nucleotide regulation of phospholipase C is illustrated in Fig. 2. In this experiment, rat pancreatic acinar cells were pre-incubated with [ $^3$ H]inositol to label the endogenous inositol lipids, and then electrically permeabilized (Baker & Knight, 1978) to permit experimental access to the intracellular side of the plasma membrane (Merritt *et al.* 1986). In this preparation, GTP- $\gamma$ -S stimulated the formation of [ $^3$ H]Ins $P_3$ . GppNHp also stimulated Ins $P_3$  formation, but was less potent. GTP itself was without effect, but this was

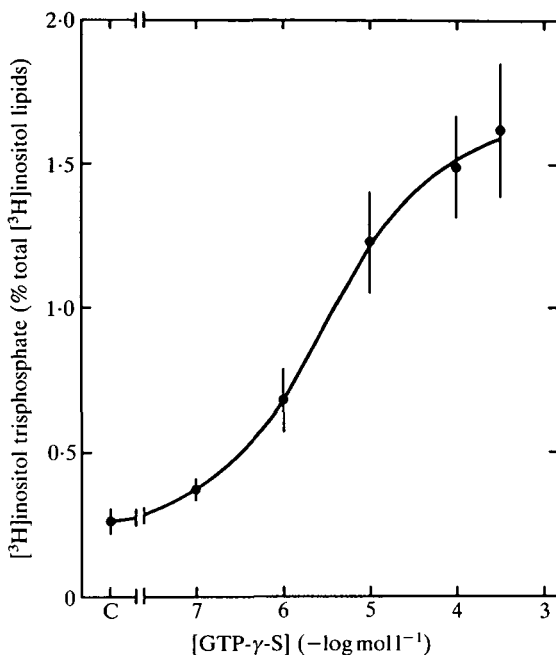


Fig. 2. GTP- $\gamma$ -S activation of polyphosphoinositide phospholipase C. Parotid acinar cells were incubated with [ $^3$ H]inositol to label the inositol lipids, then permeabilized by high-voltage electrical discharge, and incubated with the indicated concentrations of GTP- $\gamma$ -S. The amount of [ $^3$ H]InsP $_3$  formed was assayed by anion-exchange chromatography. For additional details see Taylor, Merritt, Putney & Rubin (1986), from which this is taken with permission.

probably due to the presence of saturating levels of GTP already present in the permeable cell preparation (Merritt *et al.* 1986). More importantly for establishing a role for this guanine nucleotide-mediated process in the normal receptor mechanism, the effects of the non-hydrolysable guanine nucleotides were potentiated by the phosphoinositide-linked receptor agonists, carbachol and cholecystokinin.

In many systems, receptor activation of phospholipase C is potently inhibited by pertussis toxin (Ui, 1986). Pertussis toxin is known to block the receptor-mediated inhibition of adenylate cyclase by preventing receptor activation of G $_i$  (Smigel *et al.* 1984; Taylor & Merritt, 1986). Thus, G $_i$  or (more likely) a similar protein might be involved in the regulation of phospholipase C. However, the majority of phospholipase-C-linked receptors have been shown to be insensitive to pertussis toxin, including that in the exocrine pancreas as described above (Merritt *et al.* 1986; Taylor & Merritt, 1986). Collectively, these and other experimental findings have suggested that the coupling of receptors to phospholipase C involves a guanine nucleotide-dependent regulatory protein which is similar, but not identical, to the proteins which regulate adenylate cyclase. The differential

sensitivity to pertussis toxin may indicate that different G-proteins are involved in the regulation of inositol lipid metabolism in different systems.

### Metabolism of inositol phosphates

Just 4 years ago, the formation of inositol tris- and bisphosphates in response to the activation of surface membrane receptors was demonstrated (Berridge *et al.* 1983). At this time a rather simple biochemical pathway was envisaged involving the sequential dephosphorylation of  $\text{Ins}(1,4,5)P_3$  to inositol 1,4-bisphosphate [ $\text{Ins}(1,4)P_2$ ] to inositol 1-phosphate ( $\text{Ins}1P$ ) and finally to free inositol by a lithium-sensitive inositol 1-phosphatase. Today, the picture of inositol phosphate metabolism is exceedingly complex (Putney, 1987). This is largely attributable to the power of HPLC analytical procedures that can separate inositol phosphates with only subtle structural differences.  $\text{Ins}(1,4,5)P_3$  is dephosphorylated by an extremely active 5-phosphatase to  $\text{Ins}(1,4)P_2$ , as originally suggested (Downes, Mussat & Michel, 1982), but it now seems that  $\text{Ins}(1,4)P_2$  is dephosphorylated almost exclusively to inositol 4-phosphate ( $\text{Ins}4P$ ) by a relatively non-specific inositol polyphosphate 1-phosphatase (Inhorn, Bansal & Majerus, 1987). In addition to the dephosphorylation of  $\text{Ins}(1,4,5)P_3$  by the 5-phosphatase, there exists in most tissues thus far examined a 3-kinase which transfers a phosphate from ATP to the 3 position of  $\text{Ins}(1,4,5)P_3$  to form inositol 1,3,4,5-tetrakisphosphate [ $\text{Ins}(1,3,4,5)P_4$ ] (Irvine, Letcher, Heslop & Berridge, 1986). This molecule is then, probably, dephosphorylated by the same 5-phosphatase that degrades  $\text{Ins}(1,4,5)P_3$  to form an isomeric inositol trisphosphate,  $\text{Ins}(1,3,4)P_3$ .  $\text{Ins}(1,3,4)P_3$  is then dephosphorylated by the inositol polyphosphate 1-phosphatase to inositol 3,4-bisphosphate [ $\text{Ins}(3,4)P_2$ ] (Inhorn *et al.* 1987), and to a lesser extent to  $\text{Ins}(1,3)P_2$  (Bansal, Inhorn & Majerus, 1987) by an enzyme that has not been as well characterized. These bisphosphates are then dephosphorylated primarily to a mixture of  $\text{Ins}1P$  and  $\text{Ins}3P$ , which are stereoisomers, and not resolved by conventional HPLC. The complexity of this metabolic pathway suggests that some biological function may be regulated by one of these metabolites. There is evidence for biological activity of  $\text{Ins}(1,3,4,5)P_4$  in either potentiating the action of  $\text{Ins}(1,4,5)P_3$  or modulating  $\text{Ca}^{2+}$  entry (see below).

An additional complication in the metabolism of the inositol phosphates is the demonstration that *in vitro* the soluble product of phospholipase C action on phosphatidylinositol 4,5-bisphosphate is a mixture of  $\text{Ins}(1,4,5)P_3$  and a derivative with the 1-phosphate cyclized between the 1- and 2-hydroxyls, inositol (1:2-cyclic,4,5)-trisphosphate [ $\text{Ins}(1:2\text{cyc},4,5)P_3$ ] (Connolly, Wilson, Bross & Majerus, 1986). In platelets, this  $\text{Ins}(1:2\text{cyc},4,5)P_3$  also mobilizes intracellular  $\text{Ca}^{2+}$  with about the same potency as the non-cyclic variety (Wilson *et al.* 1985); it is not a substrate for the 3-kinase, and is only slowly dephosphorylated by the 5-phosphatase (Connolly *et al.* 1987; Hawkins, Berrie, Morris & Downes, 1987). Thus, if formed *in vivo*, this compound might cause persistent activation of  $\text{Ca}^{2+}$  mobilization. In fact, reports on the production of  $\text{Ins}(1:2\text{cyc},4,5)P_3$  in cells are

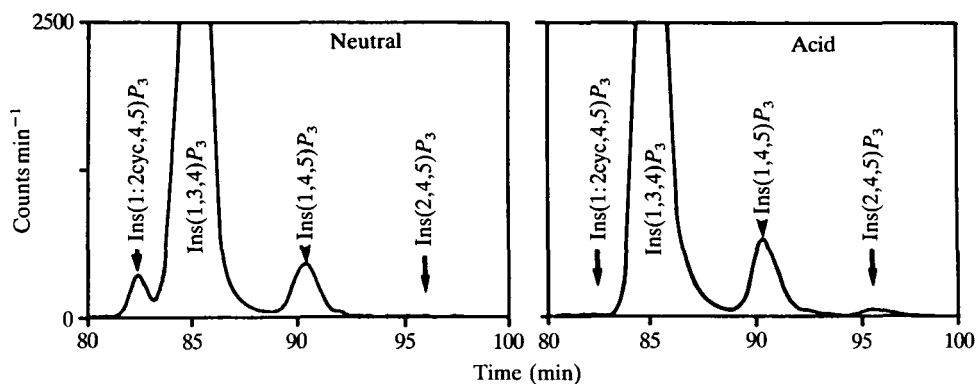


Fig. 3. HPLC analysis of [ $^3\text{H}$ ]inositol trisphosphates formed in parotid acinar cells. The data show radioactivity as a function of elution time from a Whatman Partisil SAX column. The cells were incubated with [ $^3\text{H}$ ]inositol to label the inositol lipids, and then treated with  $100\ \mu\text{mol l}^{-1}$  methacholine for 40 min. Samples extracted under neutral conditions, and then analysed by conventional strong anion-exchange HPLC, show three peaks corresponding to standards for  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(1,3,4)\text{P}_3$  and  $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$  (left). If extracted under acid conditions (right) no  $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$  is seen, the amount of  $\text{Ins}(1,4,5)\text{P}_3$  is increased and a small peak corresponding to  $\text{Ins}(2,4,5)\text{P}_3$  appears.

somewhat conflicting; it appears that on brief stimulation little of the cyclic derivative is formed (Hawkins *et al.* 1987) but, on prolonged stimulation, it accumulates (Sekar, Dixon & Hokin, 1987), presumably due to its rather slow rate of metabolism. In most published studies to date, biological samples are treated with acid prior to analysis. Under these conditions,  $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$  is converted to  $\text{Ins}(1,4,5)\text{P}_3$ , and a small amount of  $\text{Ins}(2,4,5)\text{P}_3$  (Fig. 3). Estimates from kinetics of  $\text{Ins}(1,4,5)\text{P}_3$  and  $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$  turnover in parotid gland suggest that as little as 1% of the  $\text{PtdIns}(4,5)\text{P}_2$  phospholipase C product may be cyclic (J. W. Putney, unpublished observation).

The known pathways for metabolism of inositol phosphates that arise as a result of phospholipase C activation are illustrated in Fig. 4. Inositol pentakisphosphate ( $\text{InsP}_5$ ) and hexakisphosphate ( $\text{InsP}_6$ ; phytic acid) are also present in most mammalian cells, but their levels do not change noticeably on stimulation (Heslop, Irvine, Tashjian & Berridge, 1985). This would suggest that they are not formed by phosphorylation of the known isomers  $\text{InsP}_3$  or  $\text{InsP}_4$ . Their source and physiological function are at present unknown.

### Inositol phosphates and calcium release

In virtually all systems thus far examined, receptor-activated calcium mobilization involves an initial phase of calcium release from intracellular stores, followed by a more prolonged phase of calcium entry (Putney, Poggioli & Weiss, 1981; Putney, 1987). The available evidence now strongly indicates that the internal

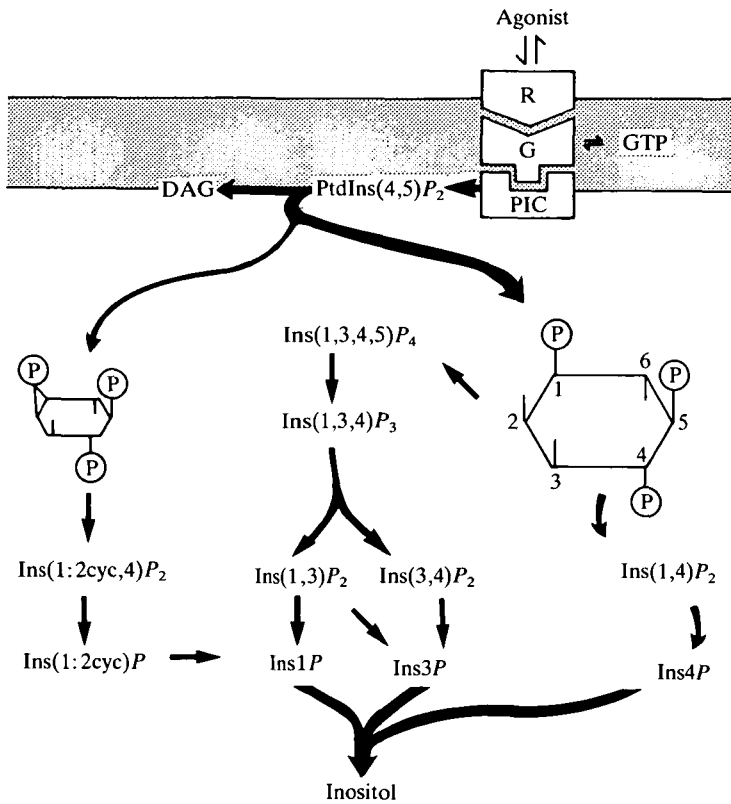


Fig. 4. Major (known) pathways for metabolism of  $\text{Ins}(1,4,5)\text{P}_3$  and  $\text{Ins}(1:2\text{cyc},4,5)\text{P}_3$ . R, receptor; G, G-protein; PIC, phosphoinositidase C; DAG, diacylglycerol;  $\text{PtdIns}(4,5)\text{P}_2$ , phosphatidylinositol 4,5-bisphosphate. See text for details.

release is signalled by  $\text{Ins}(1,4,5)\text{P}_3$  (Berridge, 1983, 1986). Thus, resolution of the site of action of  $\text{Ins}(1,4,5)\text{P}_3$  in cells would provide information on the locus of receptor-regulated calcium pools. Prior to the discovery of the second messenger function of  $\text{Ins}(1,4,5)\text{P}_3$ , a number of laboratories approached this problem by examining the distribution of total and radiolabelled calcium in subcellular fractions prepared as rapidly as possible following agonist treatment, and with precautions to minimize redistribution during fractionation. This approach was employed by several laboratories in studies of calcium-mobilizing hormones in the liver. The general finding was that, following receptor activation, mitochondrial and microsomal fractions show a net loss of calcium (Exton, 1980; Williamson, Cooper & Hoek, 1981). Other reports questioned the likelihood of mitochondrial involvement on the basis of data suggesting that normal, healthy cells do not generally contain appreciable quantities of calcium in their mitochondria (Burgess *et al.* 1983).



For  $\text{Ins}(1,4,5)P_3$  the results are more straightforward. Since the action of this mediator can be tested in permeable cells and subcellular fractions, specific poisons and other experimental manipulations can be used to determine unambiguously the contributions of mitochondrial and non-mitochondrial pools. Under these conditions, the results from a number of different laboratories concur that  $\text{Ins}(1,4,5)P_3$  induces release of calcium from an intracellular pool which is insensitive to inhibitors of mitochondrial calcium uptake and is thus likely to be a component of the endoplasmic reticulum (ER) (Streb *et al.* 1983, 1984; Burgess *et al.* 1984a,b; Joseph *et al.* 1984; Prentki, Wollheim & Lew, 1984). However, in some cells there is evidence that not all of the ER stores of calcium may be regulated by  $\text{Ins}(1,4,5)P_3$  (Taylor & Putney, 1985; Leslie, Burgess & Putney, 1988). For example, in hepatocytes, only about 30–40% of exchangeable calcium could be released by  $\text{Ins}(1,4,5)P_3$  (Burgess *et al.* 1984b). Taylor & Putney (1985) examined the effects of  $\text{Ins}(1,4,5)P_3$  on efflux of  $^{45}\text{Ca}^{2+}$  from fully loaded ER following rapid depletion of ATP by glucose and hexokinase. Under these conditions, calcium exit from the ER of the permeable cells resembles a monoexponential process with a half-time of about 5 min. This rate was dramatically increased by  $\text{Ins}(1,4,5)P_3$  such that about 40% of the  $^{45}\text{Ca}^{2+}$  content is lost in 30 s. Thereafter, in the continued presence of  $\text{Ins}(1,4,5)P_3$ , the remaining 60% is lost at a rate similar to that observed in the absence of  $\text{Ins}(1,4,5)P_3$ . This remaining fraction of accumulated calcium presumably resides in a pool, or component of the ER, which is not subject to regulation by  $\text{Ins}(1,4,5)P_3$ .

Experiments by Dawson and by Gill suggest that  $\text{Ca}^{2+}$  in the compartments of the ER insensitive to  $\text{Ins}(1,4,5)P_3$  can be released by GTP (Dawson, 1985; Dawson, Comerford & Fulton, 1986; Ueda, Chueh, Noel & Gill, 1986; Gill, Ueda, Chueh & Noel, 1986; Chueh & Gill, 1986; Chueh *et al.* 1987; Mullaney, Chueh, Ghosh & Gill, 1987). This guanine nucleotide effect is substantially different from that for receptor–effector coupling, in that non-hydrolysable analogues cannot substitute for GTP and, in fact, will block the response to GTP. Gill has shown that there are also a number of differences between the mechanism of action of GTP and that of  $\text{Ins}(1,4,5)P_3$ , most notable being the marked temperature-dependence of the GTP-induced release, and the marked temperature-independence of the  $\text{Ins}(1,4,5)P_3$ -induced release. In recent experiments, it was shown that GTP could, in fact, induce the translocation of  $\text{Ca}^{2+}$  from an  $\text{Ins}(1,4,5)P_3$ -insensitive to an  $\text{Ins}(1,4,5)P_3$ -sensitive pool (Mullaney *et al.* 1987). The ability of GTP to regulate the size of the  $\text{Ins}(1,4,5)P_3$ -sensitive pool, and the concept that it is possible to induce reversible communication between membrane-limited compartments, has profound implications and may be directly relevant to proposals discussed below on the mechanisms for receptor-regulated  $\text{Ca}^{2+}$  entry into cells.

Based on the low concentration of  $\text{Ins}(1,4,5)P_3$  necessary to release calcium, and certain structural requirements for activity, it was suggested that  $\text{Ins}(1,4,5)P_3$  acted by binding to a specific receptor on the ER (Burgess *et al.* 1984a,b). Thus, the failure of a component of the ER to respond to  $\text{Ins}(1,4,5)P_3$  may result from

the absence of the receptor in that fraction. Spat *et al.* (1986a), Spat, Fabiato & Rubin (1986b) and Baukal *et al.* (1985) prepared  $^{32}\text{P}$ -labelled  $\text{Ins}(1,4,5)\text{P}_3$  of high specific activity and demonstrated the presence of specific binding sites in microsomes and permeable cells. In permeable hepatocytes, there was a good correlation between occupancy of these sites and the mobilization of calcium by both  $\text{Ins}(1,4,5)\text{P}_3$  and  $\text{Ins}(2,4,5)\text{P}_3$ . There was also a reasonably good correlation between  $\text{Ins}(1,4,5)\text{P}_3$  binding and calcium release in rabbit neutrophils, the latter being about 10 times more sensitive to  $\text{Ins}(1,4,5)\text{P}_3$  than hepatocytes. Recently, a high-affinity binding site for  $\text{Ins}(1,4,5)\text{P}_3$  has been purified to homogeneity from bovine brain (Worley *et al.* 1987). This protein has a relative molecular mass of around 250 000, and has many of the same properties of the putative  $\text{Ins}(1,4,5)\text{P}_3$  receptor. However, no functional data are yet available to confirm the identity of this binding site with the  $\text{Ins}(1,4,5)\text{P}_3$  receptor involved in mediating intracellular release. What is sorely needed is a better selection of potent pharmacological probes to aid in the biochemical characterization of this important receptor site.

### Calcium entry

By comparison with calcium release, the regulation of the second phase of calcium mobilization, calcium entry, is poorly understood (Putney, 1986). There is strong, albeit indirect, evidence that inositol lipid metabolism is important for calcium entry (Berridge & Fain, 1979). Recently, the injection of inositol phosphates into sea urchin eggs and lacrimal gland cells produced a response pattern suggestive of an activation of both intracellular calcium release and entry of calcium from the extracellular space (Slack, Bell & Benos, 1986; Irvine & Moor, 1986, 1987; Morris, Gallacher, Irvine & Petersen, 1987). However, the direct application of  $\text{Ins}(1,4,5)\text{P}_3$  to plasma membranes does not increase their permeability to calcium (Delfert, Hill, Pershadsingh & Sherman, 1986; Ueda *et al.* 1986). Collectively, these observations suggest that  $\text{Ins}(1,4,5)\text{P}_3$  may activate calcium entry into cells, but not by acting directly on the plasma membrane.

Recently, a mechanism that is consistent with this idea has been proposed. According to this hypothesis, the emptying of an  $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool secondarily signals calcium entry. A detailed discussion of the circumstantial evidence supporting this idea has been presented elsewhere (Putney, 1986). The major evidence is derived from studies of the kinetics of emptying and refilling of a receptor-regulated calcium pool in the rat parotid gland. In the parotid gland under resting conditions, this intracellular calcium pool is resistant to depletion by extracellular chelating agents. However, when emptied by agonist stimulation, this pool can rapidly be filled from outside the cell, even in the absence of agonists and (presumably) second messengers, such as inositol phosphates (Aub, McKinney & Putney, 1982). These results suggest that the loss of calcium from this pool has somehow activated a pathway for entry into the pool from the extracellular space. Thus, in the continued presence of agonist, when  $\text{Ins}(1,4,5)\text{P}_3$  levels are being continuously maintained, the pool would presumably be held empty, the

pathway from the extracellular space would be open, and calcium would enter the ER and subsequently the cytosol through the  $\text{Ins}(1,4,5)P_3$ -activated channels. This has been termed a *capacitative* mechanism for calcium entry, because the ER is seen as behaving as a  $\text{Ca}^{2+}$  capacitance between the extracellular space and the cytosol.

Recently, evidence has been presented suggesting that  $\text{Ins}(1,3,4,5)P_4$  could act as a specific signal for calcium entry in sea urchin eggs and in lacrimal gland acinar cells (Irvine & Moor, 1986, 1987; Morris *et al.* 1987). As discussed above,  $\text{Ins}(1,3,4,5)P_4$  is formed by phosphorylation of  $\text{Ins}(1,4,5)P_3$ . In sea urchin eggs, injection of  $\text{Ins}(1,4,5)P_3$  caused full activation of the eggs (i.e. raising of a fertilization envelope), a response believed to involve intracellular calcium release as well as calcium entry (Slack *et al.* 1986). The data of Irvine & Moor (1986, 1987) show that  $\text{Ins}(2,4,5)P_3$ , an inositol phosphate that causes calcium release in other systems, but which, they argue, may not be phosphorylated to an  $\text{Ins}P_4$ , does not fully activate eggs when injected intracellularly. Also, they found that  $\text{Ins}(1,3,4,5)P_4$  did not activate eggs when injected alone. However, when  $\text{Ins}(1,3,4,5)P_4$  was injected together with the calcium-releasing inositol phosphate,  $\text{Ins}(2,4,5)P_3$ , a full fertilization response (i.e. raised envelope) was obtained. They suggest that when  $\text{Ins}(1,4,5)P_3$  is injected, it causes calcium release, but that activation of calcium entry requires its phosphorylation to  $\text{Ins}(1,3,4,5)P_4$ . But since calcium release seemed to be a prerequisite for activation of calcium entry [i.e.  $\text{Ins}(1,3,4,5)P_4$  only worked together with the calcium-releasing  $\text{Ins}(2,4,5)P_3$ ], they concluded that the emptying of the  $\text{Ins}(1,4,5)P_3$ -regulated calcium pool was necessary for activation of calcium entry by  $\text{Ins}(1,3,4,5)P_4$ . These findings may represent the first demonstrated biological action of  $\text{Ins}(1,3,4,5)P_4$ . Whether the specific mechanism suggested by Irvine & Moor (1986) is correct depends heavily on the assumptions made which were based on known properties of the inositol phosphates in other biological systems. There also seems to be no basis for attributing a specific action of  $\text{Ins}P_4$  on  $\text{Ca}^{2+}$  entry as opposed to  $\text{Ca}^{2+}$  release. In more recent experiments, Morris *et al.* (1987) demonstrated that, in perfused lacrimal acinar cells,  $\text{Ins}P_4$  was required in the perfusate to see effects of  $\text{Ins}(1,4,5)P_3$  on either the initial transient or later sustained phase of  $\text{Ca}^{2+}$  mobilization. Thus the role of  $\text{Ins}P_4$  in these cells may be a permissive one for  $\text{Ins}(1,4,5)P_3$ , and the actual biphasic mobilization of  $\text{Ca}^{2+}$  may then be accomplished through the capacitative mechanism discussed above; that is, the emptying of the  $\text{Ins}(1,4,5)P_3$ -regulated pool serves as a signal regulating calcium entry. It should be remembered that  $\text{Ins}(1,4,5)P_3$  is fully capable of activating  $\text{Ca}^{2+}$  release in permeable cells, or in microsomal preparations in the absence of  $\text{Ins}P_4$ , although it is not known whether this occurs in the concentration range actually achieved in activated cells. Recall also that, with prolonged activation of exocrine gland cells,  $\text{Ins}(1:2\text{cyc},4,5)P_3$  accumulates. If this inositol phosphate is, in fact, fully capable of mimicking the actions of  $\text{Ins}(1,4,5)P_3$ , then its slow degradation would prevent rapid restoration of basal  $\text{Ca}^{2+}$  levels in cells following the removal of a stimulus. However, if  $\text{Ins}P_4$  were necessary for the

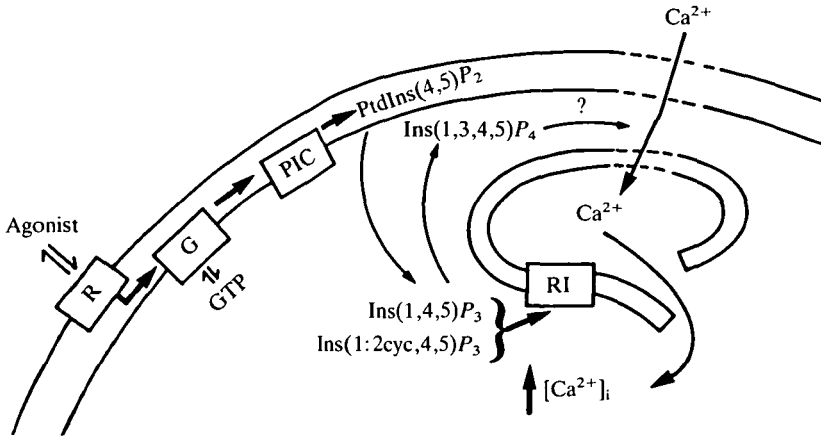


Fig. 5. Model depicting some suggested biological actions of inositol polyphosphates. Ins(1,4,5)P<sub>3</sub> and Ins(1:2cyc,4,5)P<sub>3</sub> may both be able to release Ca<sup>2+</sup> from endoplasmic reticulum, and this may secondarily signal entry of Ca<sup>2+</sup> from the extracellular space. This process may be amplified or modulated in some manner by Ins(1,3,4,5)P<sub>4</sub>; alternatively, Ins(1,3,4,5)P<sub>4</sub> may also act on the entry mechanism more directly. RI, Ins(1,4,5)P<sub>3</sub> receptor. For other abbreviations, see legend to Fig. 4.

Ins(1:2cyc,4,5)P<sub>3</sub> to act, then the rapid fall in cellular levels of InsP<sub>4</sub> would still lead to a rapid termination of the Ca<sup>2+</sup> signal. Some of the proposed roles of inositol polyphosphates in Ca<sup>2+</sup> signalling are illustrated in Fig. 5.

### Conclusions

In the past few years, our understanding of the relationships between receptors, inositol lipids and calcium has advanced at an incredible rate. Indeed, just more than 4 years before the writing of this review, Streb *et al.* (1983) first demonstrated the biological activity of Ins(1,4,5)P<sub>3</sub>; this molecule has now gained general acceptance as an important and ubiquitous second messenger. It is apparent that this second messenger system is exceedingly complex and possesses many potential sites of regulation. As with most important biochemical pathways, our knowledge raises numerous challenging questions. What is the nature of the G-protein (or proteins) that couples receptors to phospholipase C? What is the significance of the alternative pathway (phosphorylation/dephosphorylation) of Ins(1,4,5)P<sub>3</sub> metabolism? What are the nature, mechanism and locus of the Ins(1,4,5)P<sub>3</sub> receptor? How is calcium entry regulated? Indeed, how is the entire system regulated, and how does it interact with other signalling systems such as those which utilize cyclic nucleotides as messengers? We hope that the substantial progress we have made in the past few years will continue towards the resolution of these issues and also pose new questions.

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