Environmental Health Perspectives
Vol. 84, pp. 141-147, 1990

Inositol Phosphate Formation and Its Relationship to Calcium Signaling

by Arlene R. Hughes* and James W. Putney, Jr.*

The activation of a variety of cell surface receptors results in a biphasic increase in the cytoplasmic Ca^{2+} concentration due to the release or mobilization of Ca^{2+} from intracellular stores and to the entry of Ca^{2+} from the extracellular space. It is well established that phosphatidylinositol 4,5-*bis*phosphate hydrolysis is responsible for the changes in Ca^{2+} homeostasis. Stimulation of Ca^{2+}-mobilizing receptors also results in the phospholipase C-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-*bis*phosphate, with the concomitant formation of inositol (1,4,5) trisphosphate ((1,4,5)IP_3) and diacylglycerol. Analogous to the adenylyl cyclase signaling system, receptor-mediated stimulation of phospholipase C also appears to be mediated by one or more intermediary guanine nucleotide-dependent regulatory proteins. There is strong evidence that (1,4,5)IP_3 stimulates Ca^{2+} release from intracellular stores. The Ca^{2+}-releasing actions of (1,4,5)IP_3 are terminated by its metabolism through two distinct pathways. (1,4,5)IP_3 is dephosphorylated by a 5-phosphatase to inositol (1,4) *bis*phosphate; alternatively, (1,4,5)IP_3 can be phosphorylated to inositol (1,3,4,5) tetrakisphosphate by a 3-kinase. Whereas the mechanism of Ca^{2+} mobilization is understood, the precise mechanisms involved in Ca^{2+} entry are not known. A recent proposal that (1,4,5)IP_3 secondarily elicits Ca^{2+} entry by emptying an intracellular Ca^{2+} pool will be considered. This review summarizes our current understanding of the mechanisms by which inositol phosphates regulate cytoplasmic Ca^{2+} concentrations.

Introduction

An examination of the relationship of phosphoinositide turnover to Ca^{2+} signaling began in the early 1950s with the observation by Mabel and Lowell Hokin that the muscarinic cholinergic receptor agonist, acetylcholine, selectively increased the incorporation of $^{32}P_i$ into two minor plasma membrane phospholipids, phosphatidylinositol (PI) and phosphatidic acid (*1*). However, it was not until some 20 years later that Michell, noting that the receptors that stimulated phosphoinositide turnover also activated Ca^{2+}-dependent processes in the cell, proposed that receptor-stimulated phosphoinositide turnover results in a cellular Ca^{2+} response (*2*). PI is sequentially phosphorylated by kinases in the cell to phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-*bis*phosphate (PIP_2). Indeed, we now know that stimulation by any of a number of Ca^{2+}-mobilizing receptor agonists initially results in the phospholipase C-catalyzed hydrolysis of PIP_2 (*3*). Furthermore, the Hokins' initial observation of the incorporation of $^{32}P_i$ into PI results from receptor-stimulated PIP_2 hydrolysis, breakdown of the inositol phosphates to inositol, and resynthesis of PI.

Phospholipase C-catalyzed PIP_2 hydrolysis results in the formation of the water-soluble inositol (1,4,5) trisphosphate [(1,4,5)IP_3] and the lipid-soluble diacylglycerol. Berridge proposed that (1,4,5)IP_3 was the intracellular messenger that stimulated Ca^{2+} release from intracellular stores (*4*). Soon thereafter, the predicted effect of (1,4,5)IP_3 on Ca^{2+} mobilization was demonstrated; micromolar concentrations of (1,4,5)IP_3 rapidly released Ca^{2+} from a nonmitochondrial store in permeabilized pancreatic acinar cells (*5*). This result quickly was confirmed in other tissues in a number of laboratories (*6,7*). Thus, the evidence is convincing that (1,4,5)IP_3, generated upon activation of Ca^{2+}-mobilizing receptors, releases Ca^{2+} from intracellular stores.

Meanwhile, a parallel story evolved in Nishizuka's laboratory (*8-10*), which demonstrated that the other product of PIP_2 hydrolysis, diacylglycerol, also was a potent intracellular messenger. Diacylglycerol remains in the plasma membrane to activate a ubiquitous protein kinase, designated as C-kinase by Nishizuka.

This review briefly summarizes our current understanding of the mechanisms by which inositol phosphates regulate cellular Ca^{2+} metabolism. The mechanisms involved in receptor activation of

*National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.
Address reprints requests to A. R. Hughes, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.

phospholipase C, as well as the metabolic pathways by which the inositol phosphates are interconverted, are discussed. Finally, proposed mechanisms by which inositol phosphates elicit intracellular Ca^{2+} release and Ca^{2+} entry from the extracellular space are described.

Receptor Activation of Phospholipase C

The mechanisms by which cell surface receptors stimulate phospholipase C have been the focus of considerable research efforts. Much of our current understanding of these mechanisms has evolved from the well-characterized adenylyl cyclase signaling system, which converts ATP to the intracellular messenger, 3′,5′-cyclic AMP. In the adenylyl cyclase system, cell surface receptors communicate with the adenylyl cyclase enzyme, located on the cytoplasmic face of the plasma membrane, through intermediary guanine nucleotide-dependent regulatory proteins (G-proteins) (*11*). Two different G-proteins, G_s and G_i, link stimulatory and inhibitory receptors, respectively, to adenylyl cyclase.

G_s and G_i, comprised of α, β, and γ subunits, are two members of a family of heterotrimeric proteins whose function is regulated by guanine nucleotides. The α subunits of this family of G-proteins are heterogeneous, whereas the β subunits are quite similar, if not identical (*12*).

Activation of G_s by hormones (in the presence of guanine nucleotides) or by guanine nucleotides alone results in the displacement, by GTP, of the GDP bound to the α subunit and subsequent dissociation of the GTP bound-α subunit from the β/γ subunits. This activated GTP-bound-α subunit stimulates the adenylyl cyclase enzyme. GTP hydrolysis to GDP by a GTPase (an inherent activity of the G-proteins) terminates cyclic AMP formation and is assumed to result in reassociation of the G-protein subunits.

G_i also undergoes subunit dissociation after incubation with guanine nucleotides. However, the precise mechanism by which G_i inhibits adenylyl cyclase is controversial. GTP-bound α_i may directly inhibit adenylyl cyclase (*13*). Alternatively, inhibition of cyclic AMP formation may occur through the liberation of a stoichiometric excess of β/γ subunits that associate with the free α subunits of G_s, resulting in enzyme inhibition (*14*). Regardless of the precise mechanism of inhibition of adenylyl cyclase, it appears that G-protein activation requires dissociation of the protein into separate α and β/γ subunits.

The ability of the adenylyl cyclase-linked G-proteins to be purified, sequenced, and reconstituted into phospholipid vesicles was due to the capacity of these proteins to serve as substrates for covalent modification by bacterial toxins. That is, G_s and G_i are ADP-ribosylated by cholera and pertussis toxin, respectively. If this toxin-catalyzed ADP-ribosylation is performed in the presence of ^{32}P-NAD, ^{32}P-ADP-ribose is covalently transferred to G_s and G_i. The cholera toxin-catalyzed ADP-ribosylation of G_s inhibits its inherent GTPase activity to irreversibly activate G_s. On the other hand, pertussis toxin-catalyzed ADP-ribosylation of G_i inactivates the protein and blocks the receptor-mediated inhibition of adenylyl cyclase.

Receptor-mediated activation of phospholipase C also appears to involve an intermediary G-protein. The earliest indication that receptors linked to phospholipase C might occur through a G-protein(s) analogous to G_s and G_i was the observation, in a number of tissues, that guanine nucleotides decreased the apparent affinity of agonists for receptors known to stimulate phospholipase C (*15–21*).

The subsequent observation that guanine nucleotide analogs potentiated the stimulatory actions of thrombin on diacylglycerol formation, protein phosphorylation, and serotonin secretion in permeabilized platelets provided more direct evidence of the involvement of a G-protein in the receptor activation of phospholipase C (*22,23*). Most recently, several laboratories have demonstrated a guanine nucleotide-mediated activation of phospholipase C in membrane or permeable cell preparations from a number of cell types (*24–29*). The predicted synergistic stimulation of PIP_2 hydrolysis by agonists and guanine nucleotides also has been observed (*29,30*). Furthermore, activation of phospholipase C by guanine nucleotides shows the same relative sensitivity to guanine nucleotide analogs as was observed with the adenylyl cyclase signaling system (*30*).

Taken together, the guanine nucleotide dependence of receptor-stimulated phosphoinositide turnover in permeabilized cells or in membrane preparations, the hormonal stimulation of GTPase activity (*19*), and the guanine nucleotide regulation of agonist binding to the Ca^{2+}-mobilizing receptors suggest a striking similarity between the G-protein that links cell surface receptors to phospholipase C and the G-proteins that couple receptors to the activation and inhibition of adenylyl cyclase. However, unlike the adenylyl cyclase system, the precise identity of the G-protein(s) mediating phospholipase C activation is currently unknown.

Two general mechanisms have been described for receptor activation of phosphoinositide turnover. First, in some systems (including neutrophils and mast cells), receptor activation of phosphoinositide turnover is sensitive to inactivation by pertussis toxin (*31–33*). This result suggests that G_i or (more likely) a G_i-like protein regulates phospholipase C in these cells. However, stimulation of inositol phosphate formation by the majority of phospholipase C-linked receptors is insensitive to pertussis toxin (*30,34,35*). This result is consistent with the hypothesis that a guanine nucleotide-dependent regulatory protein that is similar, but not identical to the proteins that regulate adenylyl cyclase, links cell surface receptors to

phospholipase C. This differential sensitivity to pertussis toxin may indicate that different G-proteins regulate phosphoinositide metabolism in different tissues.

Recently, several toxin-insensitive G-proteins have been identified and/or purified based on their capacity to bind radiolabeled guanine nucleotides with high affinity and on the inability of these proteins to serve as substrates for ADP-ribosylation by pertussis toxin (*36–38*). However, to date, the purification and successful reconstitution of a pertussis toxin-insensitive G-protein linked to phospholipase C has not been achieved.

Inositol Phosphate Metabolism

Inositol phosphates were initially envisioned to be metabolized through a single, simple pathway that involved the sequential dephosphorylation of (1,4,5)IP_3 to (1,4)IP_2, (1)IP and inositol. However, with the advent of powerful HPLC analytical procedures that resolve inositol phosphates with only subtle structural differences, we now know that inositol phosphate metabolism is exceedingly complex (Fig. 1). The Ca^{2+}-mobilizing actions of (1,4,5)IP_3 are terminated by its rapid dephosphorylation to (1,4)IP_2 by a 5-phosphatase (*39*). (1,4)IP_2 appears to be dephosphorylated solely to (4)IP by a relatively nonspecific inositol polyphosphate 1-phosphatase (*40*). (4)IP is hydrolyzed by a lithium-sensitive inositol monophosphatase to inositol (*41*).

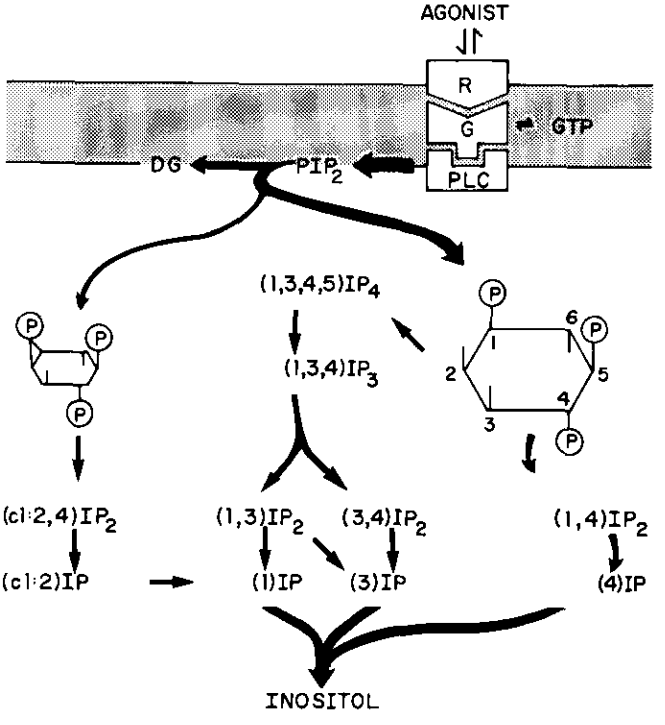

FIGURE 1. Metabolic pathways of the inositol phosphates generated upon phospholipase C-catalyzed hydrolysis of phosphatidylinositol 4,5-*bis*phosphate (PIP_2).

In addition to dephosphorylation of (1,4,5)IP_3 by the 5-phosphatase, (1,4,5)IP_3 is metabolized through a second route. In most tissues thus far examined, a 3-kinase exists that phosphorylates the 3-position of (1,4,5)IP_3 to form (1,3,4,5)IP_4 (*42*). (1,3,4,5)IP_4 is dephosphorylated to a different inositol trisphosphate, (1,3,4)IP_3—presumably by the same 5-phosphatase that dephosphorylates (1,4,5)IP_3. (1,3,4)IP_3 is dephosphorylated primarily to (3,4)IP_2 by the inositol polyphosphate 1-phosphatase (*40*), and to a lesser extent, to (1,3)IP_2 (*43*). These inositol *bis*phosphates are metabolized to a mixture of (1)IP and (3)IP, which are stereoisomers, and not resolved by conventional HPLC methods. The complexity of the metabolism of (1,4,5)IP_3 suggests that inositol phosphates, in addition to (1,4,5)IP_3, may be biologically active in the cells. Recent evidence, detailed below, suggests that (1,3,4,5)IP_4, together with (1,4,5)IP_3 may modulate Ca^{2+} entry.

An additional complication in inositol phosphate metabolism is the observation, *in vitro*, that purified phospholipase C-catalyzed hydrolysis of PIP_2 results in the formation of (1,4,5)IP_3 and cyclic (1:2,4,5)inositol trisphosphate ((cl:2,4,5)IP_3), a derivative with the 1-phosphate cyclized to the 2-hydroxyl (*44,45*). (cl:2,4,5)IP_3 mobilizes intracellular Ca^{2+} from permeabilized platelets (*46*) and 3T3 cells (*47*) with a potency similar to that of noncyclic (1,4,5)IP_3. However, (cl:2,4,5)IP_3 is not a substrate for the 3-kinase, and it is degraded by the 5-phosphatase more slowly than (1,4,5)IP_3 (*48–50*). The slower inactivation of (cl:2,4,5)IP_3 suggests that if it is formed *in vivo*, it may cause persistent activation of Ca^{2+} mobilization.

In cholinergically stimulated pancreatic minilobules, Dixon and Hokin determined that the levels of (1,4,5)IP_3 rapidly increased, then fell to a new elevated steady-state, whereas (cl:2,4,5)IP_3 slowly accumulated (*51*). Furthermore, they suggested that (1,4,5)IP_3 could be responsible for intracellular Ca^{2+} release at short times of stimulation, whereas both (1,4,5)IP_3 and (cl:2,4,5)IP_3 might contribute equally to Ca^{2+} release during prolonged stimulation. However, in most published reports of inositol phosphate formation *in vivo*, experiments are terminated by the addition of acid, which cleaves the 1:2 cyclic bond to yield a mixture of (1,4,5)IP_3 and (2,4,5)IP_3. To date, an accurate assessment of the amounts of (cl:2,4,5)IP_3 formed in cells, as well as its contribution to the Ca^{2+} response in a variety of tissues, has not been determined.

Inositol pentakisphosphate (IP_5) and inositol hexakisphosphate (IP_6, phytic acid) also are present in most mammalian cells. Their levels do not seem to change upon receptor stimulation (*52*), suggesting that they are not formed by phosphorylation of (1,4,5)IP_3 or (1,3,4,5)IP_4. Their source and function are currently unknown, although Vallejo et al. recently demonstrated that picomolar amounts of IP_5 and IP_6 elicited marked cardiovascular effects when injected into specific brain stem regions (*53*). They suggested

that IP_5 may have extracellular sites of action. Recently, two novel inositol tetrakisphosphates have been identified—(1,3,4,6)IP_4 which is formed from the phosphorylation of (1,3,4)IP_3 (*54,55*), and D-(3,4,5,6)IP_4 (*56*) whose metabolic source is unknown. These compounds are potential precursor (or metabolite) candidates for IP_5 and IP_6.

Inositol Phosphates and Ca^{2+} Release

It is well established that receptor-stimulated Ca^{2+} mobilization involves an initial release of Ca^{2+} from intracellular stores, followed by Ca^{2+} entry from the extracellular space (*57,58*). This biphasic Ca^{2+} response can be measured either directly by the fluorescent Ca^{2+} indicators, Quin-2 and Fura-2 (*59*), or indirectly by monitoring changes in the Ca^{2+}-dependent processes [such as unidirectional $^{86}Rb^+$ efflux through Ca^{2+}-activated K^+ channels (*60*)].

Specifically, the addition of a Ca^{2+}-mobilizing agonist results in a rapid and transient increase in cytoplasmic Ca^{2+}, which persists in the absence of extracellular Ca^{2+} and, therefore, appears to result from the release of an intracellular pool of Ca^{2+}. A sustained phase then follows, which is dependent on the presence of extracellular Ca^{2+} and presumably reflects Ca^{2+} entry from the extracellular space. There is strong evidence to support the proposal that (1,4,5)IP_3 mediates intracellular Ca^{2+} release (*61*).

The effects of (1,4,5)IP_3 were examined in permeabilized cells and in subcellular fractions under experimental conditions that would selectively poison mitochondrial versus nonmitochondrial pools. These manipulations led a number of laboratories to conclude that (1,4,5)IP_3 releases Ca^{2+} from an intracellular pool that was insensitive to inhibitors of mitochondrial Ca^{2+} uptake and, by default, was likely to be a component of the endoplasmic reticulum (*5-7,62,63*). However, it appears that only a portion of the Ca^{2+} stored in the endoplasmic reticulum is released by (1,4,5)IP_3. Approximately 30 to 40% of the exchangeable Ca^{2+} in the endoplasmic reticulum of permeabilized hepatocytes is releasable by (1,4,5)IP_3 (*6,64*), which suggests that the remaining Ca^{2+} is present in a pool not regulated by (1,4,5)IP_3.

Ca^{2+} Entry

Whereas the evidence is quite convincing that (1,4,5)IP_3 is the intracellular signal that mobilized intracellular Ca^{2+}, the second phase of the Ca^{2+} response—namely, Ca^{2+} entry—is not well understood. The inositol phosphates, particularly (1,4,5)IP_3, also may be important in Ca^{2+} entry. Microinjection of inositol phosphates into sea urchin eggs or lacrimal cells results in cell responses requiring both Ca^{2+} release and Ca^{2+} entry (*65-67*). In addition, the application of (1,4,5)IP_3 to excised patches of T lymphocytes elicits Ca^{2+} channel activity similar to that observed by the addition of agonist, suggesting that (1,4,5)IP_3 activates Ca^{2+} channels in the plasma membrane (*68*).

However, in other systems, (1,4,5)IP_3 does not directly increase the permeability of plasma membrane vesicles to Ca^{2+} (*69,70*). This suggests that if (1,4,5)IP_3 promotes Ca^{2+} entry into cells, the site of action of (1,4,5)IP_3 is not at the plasma membrane.

Recently, a capacitative mechanism was proposed in which (1,4,5)IP_3 secondarily promotes Ca^{2+} entry (*71*), by emptying intracellular Ca^{2+} stores. We know that the ability of a cell to respond to a second Ca^{2+}-mobilizing agonist, following the termination of the first stimulus, depends on the refilling of the IP_3-sensitive intracellular pool (*60,72*). Under resting conditions, this IP_3-sensitive intracellular Ca^{2+} store is resistant to depletion by extracellular chelating agents. However, upon depletion, refilling of this pool only occurs in the presence of extracellular Ca^{2+}. This refilling process occurs after the termination of the first stimulus (and presumably in the absence of intracellular messengers, such as inositol phosphates). This intracellular pool appears to be in close apposition to the plasma membrane because the refilling process occurs with only a small increase in the cytoplasmic Ca^{2+} concentration (*59*). According to this capacitative model, emptying of the intracellular Ca^{2+} pool by IP_3 permits the direct communication of this pool with the plasma membrane. In the presence of extracellular Ca^{2+}, Ca^{2+} enters the cell through this interface and subsequently into they cytosol. When IP_3 is degraded, extracellular Ca^{2+} continues to enter the cell via this interface until the intracellular Ca^{2+} pool is restored.

As previously mentioned, (1,3,4,5)IP_4 possesses no Ca^{2+}-releasing activity (*47*); thus, the metabolism of (1,4,5)IP_3 to (1,3,4,5)IP_4 simply may terminate the actions of (1,4,5)IP_3. However, recent evidence suggests that (1,3,4,5)IP_4 may be important in the second phase of the cellular Ca^{2+} response, namely Ca^{2+} entry. In sea urchin eggs, the injection of (1,4,5)IP_3 results in cell activation (the raising of a fertilization envelope), a response believed to require both Ca^{2+} release as well as Ca^{2+} entry (*65,66*).

Irvine and Moor demonstrated that the injection of (2,4,5)IP_3, which releases intracellular Ca^{2+} but which presumably is not phosphorylated to IP_4 (*50*), resulted in the activation of many fewer cells (*66*). Similarly, microinjection of (1,3,4,5)IP_4 alone failed to raise a fertilization envelope. However, when (2,4,5)IP_3, which releases Ca^{2+}, was injected together with (1,3,4,5)IP_4, a full cellular response was observed. They suggested that when (1,4,5)IP_3 is injected, it causes Ca^{2+} release, but that activation of Ca^{2+} entry requires its phosphorylation to (1,3,4,5)IP_4. However, since (1,3,4,5)IP_4 only was active when injected with the Ca^{2+}-releasing (2,4,5)IP_3, they concluded that the

emptying of the intracellular Ca^{2+} pool appeared to be a prerequisite to Ca^{2+} entry. Furthermore, the role of $(1,4,5)IP_3$ could not be mimicked by artificially raising the cytoplasmic Ca^{2+} concentration (*73*). Thus, it appears that in intact cells, both $(1,4,5)IP_3$ and $(1,3,4,5)IP_4$ levels must increase for the full Ca^{2+} response.

In more recent experiments, Morris et al. (*67*) demonstrated that in perfused lacrimal acinar cells, $(1,3,4,5)IP_4$ was required in the perfusate to observe effects of $(1,4,5)IP_3$ on either the initial transient or later sustained phase of Ca^{2+} mobilization. This might indicate that $(1,3,4,5)IP_4$ is required for $(1,4,5)IP_3$-induced Ca^{2+} release as well as entry. Somewhat paradoxically, $(1,4,5)IP_3$ alone is fully capable of emptying intracellular Ca^{2+} stores when applied to microsomal preparations or permeable cells (which would correspond to the initial transient phase of the Ca^{2+} response in the lacrimal cells). Thus, despite the striking effects of $(1,3,4,5)IP_4$ demonstrated in the sea urchin egg and lacrimal cell experiments, the precise mechanism by which $(1,3,4,5)IP_4$ acts in the Ca^{2+}-signaling pathway remains to be resolved.

Conclusions

Virtually our entire understanding of the mechanisms by which the inositol phosphates regulate the Ca^{2+}-signaling system has evolved within the last 5 years. Whereas $(1,4,5)IP_3$ has been accepted as the signal that elicits intracellular Ca^{2+} release, the second phase of the cellular Ca^{2+} response, namely Ca^{2+} entry, is less understood. Furthermore, the complexity of the metabolism of the inositol phosphates (with its alternative phosphorylation/dephosphorylation pathways) implies that additional inositol phosphates may be biologically active. It seems safe to predict that as our knowledge of the phosphoinositide/Ca^{2+}-signaling system increases, our unanswered questions will increase as well.

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