The Metabolism of Phosphoinositide-Derived Messenger Molecules

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The phosphoinositides are minor phospholipids present in all eukaryotic cells. They are storage forms for messenger molecules that transmit signals across the cell membrane and evoke responses to extracellular agonists. The phosphoinositides break down to liberate messenger molecules or precursors of messenger molecules. Many different compounds are formed, although the functions of only a few are understood. Recent studies elaborating the pathways for formation of products from phosphoinositides and the factors controlling their metabolism are summarized here.

THERE HAS BEEN A GREAT DEAL OF PROGRESS IN RECENT years in the understanding of mechanisms by which cells respond to extracellular signals. These extracellular signals are transmitted across the cell membrane by a variety of mechanisms that utilize messenger molecules. Hormones, peptide growth factors, neurotransmitters, and other impermeant agonists bind to specific receptors on the external surface of a cell. Occupancy of these receptors initiates the production of active messengers, including the well-studied cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) molecules, as well as the more recently discovered messenger molecules that are derived from phosphoinositides. Messengers, once formed, evoke a host of intracellular and cell-to-cell reactions. We have summarized here recent results from our laboratory on the production and metabolism of the inositol phosphates. Many general review articles on the physiology of phosphoinositide-derived messenger molecules have appeared in the last few years (*1*). The full nature and significance of many of these compounds is not understood, although at least three different messenger molecules are known to be produced from phosphoinositides—arachidonic acid, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], and 1,2-diacylglycerol. Arachidonate is oxygenated to form other mediators including prostaglandins, thromboxanes, and leukotrienes. Ins(1,4,5)P_3 functions as a messenger to mobilize Ca^{2+} from an intracellular site, probably in the endoplasmic reticulum, while diacylglycerol is a messenger that acts as an essential cofactor for protein kinase C.

Phosphoinositides constitute 2 to 8% of the lipid in cell membranes in eukaryotic cells and are essential for cell survival (*2*). The polar head group of these lipids is *myo*-inositol, as shown for phosphatidylinositol (PtdIns) in Fig. 1. A minority of the molecules contain additional phosphate groups as monoesters either in the 4 position of inositol (phosphatidylinositol 4-monophosphate) (PtdIns4P) or in both the 4 and 5 positions of inositol (phosphatidylinositol 4,5-bisphosphate) [PtdIns(4,5)P_2]. These polyphosphoinositides are formed from PtdIns by kinases and are degraded back to PtdIns by phosphatases, as shown in the upper layer of Fig. 2. In most cells the polyphosphoinositides represent 10 to 20% of the inositol lipids. PtdIns(4,5)P_2 is usually less prevalent than PtdIns4P, and thus the PtdIns(4,5)P_2 content of cells is only 1 to 10% that of the PtdIns content. Although the function of the phosphoinositides is only now being elucidated, their structures and routes of biosynthesis were elucidated around 1960. Kennedy and co-workers defined the pathway for PtdIns biosynthesis in microsomes (*3*), and the structures of the polyphosphoinositides were established in a classical series of studies by Ballou and co-workers (*4*). Indeed, the methods that Ballou developed remain important tools in defining the structures of the newly discovered inositol phosphates described below.

We have only recently begun to appreciate that the phosphoinositides serve as storage forms of messenger molecules. Hokin and Hokin first discovered that the phosphoinositides are more actively metabolized than other lipids (*5*). They and others subsequently showed that a large number of tissues displayed accelerated phosphoinositide metabolism when stimulated by various agonists (*6*). Durell (*7*), and later Michell (*6*), suggested that accelerated phosphoinositide turnover was a response of cells that reflected the transduction of signals across the cell membrane. Michell also noted that accelerated phosphoinositide turnover was linked with cellular processes that are associated with Ca^{2+} mobilization. He therefore proposed that phosphatidylinositol turnover might in some way trigger Ca^{2+} mobilization. These predictions have been borne out in part by recent experiments.

Production of Messengers from Phosphoinositides

Our current view of the main pathways of phosphoinositide metabolism is shown in Fig. 2. Phosphoinositides break down rapidly in response to occupancy of several types of receptors by specific agonists (*1*). The three phosphoinositides are degraded by a phosphoinositide-specific phospholipase C (PLC) to form diacylglycerol (1,2-DG) and the various inositol phosphates (Fig. 2). The inositol phosphates are rapidly degraded to inositol (I), which is utilized for resynthesis of phosphoinositides. Diacylglycerol either is hydrolyzed by lipases to monoacylglycerol (2MG) and then to free

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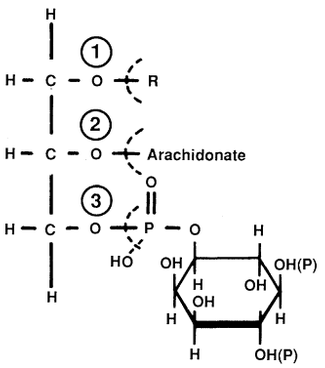


Fig. 1. Structure of phosphatidylinositol. The polyphosphoinositides contain additional monoester phosphates in the 4 position (opposite the phosphodiester) or in both 4 and 5 positions. The breakdown of PtdIns is initiated by PLC cleavage of bond 3. The diacylglycerol can be further cleaved by lipases with bond 1 preceding bond 2.

arachidonate and glycerol or is phosphorylated by diacylglycerol kinase to form phosphatidic acid (PA), which is then used in the synthesis of phosphoinositides.

Most early studies of phosphoinositide metabolism were confined to PtdIns itself. However, it is clear that the polyphosphoinositides are also degraded by PLC (*8*). A single PLC isolated from ram seminal vesicles can cleave all three phosphoinositides in vitro (*9*). In stimulated cells radiolabeled with [^{3}H]inositol, Ins(1,4,5)P_3 and inositol 1,4-bisphosphate [Ins(1,4)P_2] appear before inositol 1-phosphate [Ins 1P], thereby indicating that polyphosphoinositide breakdown is the initial agonist-stimulated event in PtdIns turnover (*10*). On the basis of these findings, Berridge has proposed a "bifurcating signal pathway" wherein, in contrast to the scheme shown in Fig. 2, all phosphoinositide breakdown proceeds by phospholipase C action on PtdIns(4,5)P_2. According to this idea, Ins(1,4)P_2 and Ins 1P are derived from phosphatase action on Ins(1,4,5)P_3 (*11*). Therefore 1 mol of Ins(1,4,5)P_3 is produced per mole of diacylglycerol. This proposal has been supported by a number of studies in which the kinetics of inositol phosphate metabolism have been measured radioisotopically (*12*). However, this proposal is inconsistent with a number of recent findings.

In platelets stimulated by thrombin, half of the total cellular PtdIns is broken down within 90 seconds. The initial mass of PtdIns is 14 times greater than that of PtdIns(4,5)P_2. Thus, if the bifurcating signal pathway hypothesis is correct, PtdIns(4,5)P_2 must turn over many times within the 90 seconds of thrombin stimulation. When platelets are incubated for 3 minutes with $^{32}PO_4$ before stimulation, the phosphoinositides are labeled to different specific activities. Under these nonequilibrium conditions, the time course of change in specific activity reflects turnover. The rise in specific activity of PtdIns4P is similar in stimulated and unstimulated cells, indicating that there is little increase in the conversion of PtdIns to PtdIns4P during thrombin stimulation. In addition, the specific activity of the phosphate in the 4 position in PtdIns4P during thrombin stimulation is less than both the phosphate in the 5 position of PtdIns(4,5)P_2 and the phosphate group of phosphatidic acid, indicating that the 4-position phosphate moiety is not labeled to equilibrium with adenosine triphosphate (ATP). This finding is inconsistent with a rapid flux of PtdIns via PtdIns4P to PtdIns(4,5)P_2 during thrombin stimulation, in which case the 4-position phosphate would be at maximum specific activity. We therefore conclude that the bulk of PtdIns breakdown that occurs in thrombin-stimulated platelets occurs by direct PLC hydrolysis of PtdIns (*13*). These results have been extended by Verhoeven and coworkers who reached the same conclusion using different methods (*14*). Imai and Gershengorn (*15*) have studied the kinetics of phosphoinositide turnover in thyrotropin releasing hormone–stimulated rat pituitary cells. They find that breakdown of PtdIns(4,5)P_2 lasts for less than 2 minutes but PtdIns breakdown persists for 30 minutes. This indicates that PLC acts on both phosphoinositides within these cells but with different time courses. Several other studies report direct breakdown of PtdIns4P and PtdIns (*16*). Recently, Rittenhouse and Sasson (*17*) measured the mass of Ins(1,4,5)P_3 produced in thrombin-stimulated platelets. They found approximately 0.2 nmol of Ins(1,4,5)P_3 formed per 10^9 platelets 15 seconds after the addition of thrombin. This compares to approximately 10 nmol of PtdIns degraded during this time, indicating that the Ins(1,4,5)P_3 production is inadequate to account for PtdIns breakdown. A difference between Ins(1,4,5)P_3 and diacylglycerol production has been reported in stimulated hepatocytes (*18*). Taken together, these studies indicate that all three phosphoinositides are utilized directly by PLC. A potential physiological consequence of the independent breakdown of phosphoinositides is that it allows for separate regulation of the production of Ins(1,4,5)P_3 and diacylglycerol, which are messenger molecules that serve different functions. The very complexity of the system allows the possibility that other signal-generating molecules may be formed.

Icosanoid messengers. The production of icosanoid mediators (oxygenated derivatives of arachidonic acid and related polyunsaturated fatty acids, including prostaglandins, thromboxanes, and leukotrienes) begins with the release of arachidonate from phospholipids (*19*). Arachidonate release in all tissues that produce icosanoids is associated with accelerated phosphoinositide turnover. In fact, arachidonate is derived in part from phosphatidylinositol itself by a series of reactions involving PLC, and diacylglycerol and monoacylglycerol lipases (*20*).

$$\text{PtdIns} \xrightarrow[\text{PLC}]{} \text{1,2-DG} \xrightarrow[\text{1,2-DG lipase}]{} \text{2MG} \xrightarrow[\text{2MG lipase}]{} \begin{matrix}\text{arachidonate}\\ + \\ \text{glycerol}\end{matrix}$$

The fraction of arachidonate released from PtdIns versus other phospholipids varies widely, depending on the cell type and the stimulus (*19, 21*). The release of arachidonic acid from other lipids occurs after PtdIns turnover in most cases and may be in some way triggered by it.

Arachidonate is liberated also by phospholipase A_2 (*22*). In most cell types, it is difficult to estimate accurately the source of arachidonate because a small fraction of the total cell arachidonate in any particular phospholipid is liberated. After incubation of platelets with low concentrations of thrombin for 5 minutes, or at early times after stimulation with high concentrations of thrombin (less than 15 seconds), arachidonate is liberated primarily from PtdIns (*23*). Phosphatidylcholine provides most of the arachidonate that is liberated after a maximal thrombin stimulation (*24*). Approximately 50% of the total platelet PtdIns breaks down in response to thrombin through the action of PLC. The diacylglycerol formed is rapidly metabolized; one-third is converted to phosphatidic acid (*25*). If the remaining diacylglycerol is degraded by lipases, this would account for approximately one-third of the total of the arachidonate released. Mohadevappa and Holub (*26*) have measured the release of saturated fatty acids derived from the 1 position of diacylglycerol and concluded that about 30% is degraded by lipases in platelets. There is some uncertainty in these measurements because the rate of turnover of the saturated fatty acids is not known and inhibitors were used in these experiments to block the metabolism of arachidonate.

Phospholipase C

Phosphoinositide-specific phospholipase C is the enzyme that generates phosphoinositide-derived messenger molecules. It cleaves phosphoinositides to yield 1,2-diacylglycerol and inositol phos-

phates. The enzyme is present in most cell types and most of the activity is cytosolic, although its substrates are in a membrane bilayer. Membrane-bound PLC has also been described and will be discussed below. Two distinct soluble phospholipase C enzymes have been identified in ram seminal vesicles (*27*). The first enzyme, designated PLC-I, was purified to homogeneity from this tissue. The pure enzyme has a specific activity of approximately 30 μmol of PtdIns hydrolyzed per minute per milligram of protein. When assayed in the absence of detergents, the enzyme has a *p*H optimum of 5.3, which shifts to nearly *p*H 7.0 in the presence of deoxycholate. The molecular weight of the enzyme is 65,000, and it is a single polypeptide chain that is not glycosylated. Upon chromatography on ion exchange columns, such as aminohexylagarose, the enzyme is resolved into two peaks. The basis for this heterogeneity is unknown.

The second enzyme from seminal vesicles, designated PLC-II, has a molecular weight of 85,000 and, although it is not completely homogeneous, it appears to have an activity similar to PLC-I. Antibodies raised against each of these enzymes do not react with the other. The tissue distribution of the enzymes appears to differ—PLC-I is the predominant form in liver, and PLC-II is the major form in platelets and brain. Multiple forms of soluble PLC also occur in partially purified preparations from other tissues (*28*). The relationship between these multiple forms of enzyme and those described above are unclear. Low (*29*) reports that platelets have three forms of PLC made up of two different types of peptides of molecular weight 140,000 and 95,000. Banno and co-workers (*30*) also report three different platelet enzymes of molecular weight 120,000, 70,000, and one, which has been purified, of 65,000—similar to that of seminal vesicle PLC-I. Another PLC that has been purified from liver and platelets has a molecular weight of 65,000 (*31*). The reported specific activities of these enzymes range from 1 to 10% that of the enzymes from seminal vesicles.

Substrate specificity. The PLC enzymes are specific for PtdIns and the polyphosphoinositides (see below). They do not hydrolyze other phospholipids with the exception of phosphatidylglycerol, which is utilized 0.001 times as well as PtdIns (*27*). The fact that the enzymes in in vitro assays require Ca^{2+} to hydrolyze PtdIns readily distinguishes them from lysosomal PLC, which is not specific for PtdIns nor inhibited by Ca^{2+} chelation. The PLC enzymes from seminal vesicles do not show specificity for particular fatty acid–containing substrate molecules. The 2 position fatty acid is completely irrelevant since 1-acyl, 2-lyso PtdIns is hydrolyzed at the same rate as PtdIns. A study with platelet PLC also indicates no fatty acid preference of the enzyme (*32*).

Both PLC-I and PLC-II readily hydrolyze all three phosphoinositides (*9*). When the three lipids are incorporated into unilamellar vesicles, they compete with each other for PLC. When present in equimolar proportions, hydrolysis of the polyphosphoinositides is favored. Increasing the proportion of PtdIns or Ca^{2+} concentration favors hydrolysis of PtdIns.

Control of Phospholipase C Activity

Membrane lipids can regulate phospholipase C activity. Although these PLC enzymes are soluble, they are inactive unless bound to a lipid bilayer containing an appropriate substrate. This is evident from a study of phosphonate analogues of PtdIns that were investigated as potential inhibitors of PLC (*33*). Derivatives too polar to insert into lipid bilayers, such as diacetyl or dibutyryl, did not inhibit or compete with PtdIns as substrate for the enzyme (the derivatives used were 3,4-diacyloxybutylphosphonyl-*myo*-inositols). Although the enzymes readily hydrolyze PtdIns in detergent dispersions or unilamellar vesicles with mixtures of PtdIns and phosphatidylethanolamine (PE), they are nearly inactive when tested with natural membranes or lipids extracted from cell membranes. This poor activity is due largely to the high content of phosphatidylcholine (PC) in membranes, which markedly inhibits PLC. It shields the substrates from the enzyme and does not allow the enzyme to bind to PC-containing PtdIns vesicles (*9, 34*). PC inhibits polyphosphoinositide hydrolysis less than PtdIns hydrolysis. The inhibition of PLC activity by PC can be reversed by phosphatidylserine, diacylglycerol, or free fatty acids. Whether these substances play any role in the control of PLC in vivo is unknown. It is interesting that these same substances (diacylglycerol, phosphatidylserine, and free fatty acid) also stimulate protein kinase C activity (*35*). This latter enzyme is also cytosolic in location and is active only when bound to membranes. In the case of protein kinase C, the stimulation by diglyceride is specific for 1,2-diacylglycerol. Neither 1,3- nor 2,3-diacylglycerol is active (*36*). PLC, in contrast, is stimulated by all diglycerides.

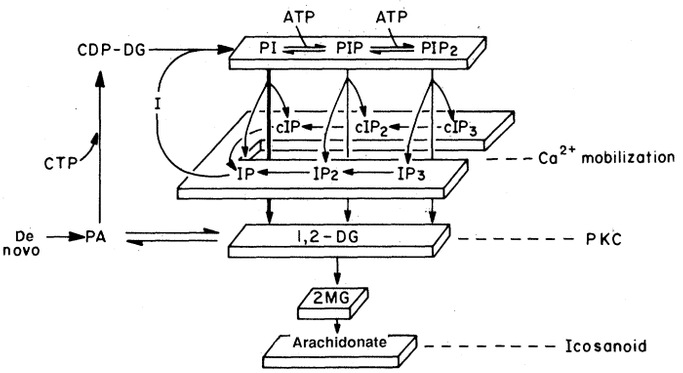


Fig. 2. Scheme for phosphoinositide breakdown and resynthesis. PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; cIP_3, inositol 1:2-cyclic 4,5-trisphosphate; IP_2, inositol 1,4-bisphosphate; cIP_2, inositol 1:2-cyclic 4-bisphosphate; IP, inositol 1-phosphate; cIP, inositol 1:2-cyclic phosphate; 1,2-DG, 1,2-diacylglycerol; PA, phosphatidic acid; CDP-DG, cytidine diphosphate diacylglycerol; I, inositol; 2MG, 2 monoacylglycerol, PKC, protein kinase C. Messengers derived from phosphoinositides are shown on the right.

The Ca^{2+} concentration determines both the rate of hydrolysis of PtdIns and the preferred substrate in in vitro assays. Unilamellar vesicles made from total platelet lipids have a ratio of PtdIns to PtdIns(4,5)P_2 of approximately 14. PLC from platelets cleaves PtdIns 20 times faster than PtdIns(4,5)P_2 with these vesicles at 100 μ*M* Ca^{2+} concentration. In contrast, at 0.1 μ*M* Ca^{2+}, the two substrates are cleaved equally well even though the concentration of PtdIns is 14 times higher. Although Ca^{2+} stimulates a severalfold increase in polyphosphoinositide hydrolysis in in vitro reactions, the reaction proceeds even in the presence of EGTA (*9*). The finding that PtdIns(4,5)P_2 hydrolysis occurs at low Ca^{2+} is consistent with the theory that this reaction triggers Ca^{2+} mobilization in the cell. PtdIns breakdown requires Ca^{2+} (the K_m is approximately 1 μ*M*) in in vitro assays and presumably the Ca^{2+} flux stimulated by Ins(1,4,5)P_3 initiates PtdIns hydrolysis. However, the role of Ca^{2+} in controlling PtdIns hydrolysis in intact cells is uncertain since raising Ca^{2+} with drugs (Ca^{2+} ionophores) does not always result in PtdIns hydrolysis (*37*).

In platelets, increased intracellular levels of cAMP block phosphoinositide breakdown, arachidonate release, and secretion of granule contents in response to agonists. The mechanism by which cAMP blocks phosphoinositide turnover is unknown. An inhibition of PLC by cAMP has been proposed (*38*), although cAMP has no direct effect on purified PLC as assayed in vitro (*39*). Alternatively,

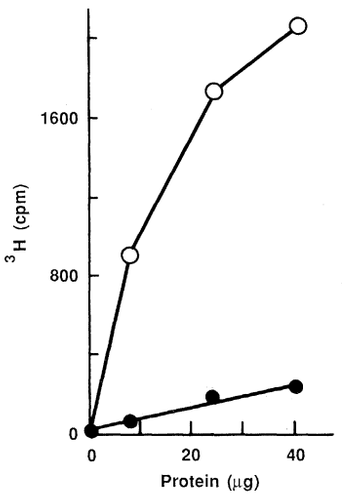


Fig. 3. Effect of GTPγS on phospholipase C activity. PLC was measured with [^{3}H]PtdIns(4,5)P_2 as the substrate as a function of platelet cytosol protein, either with (○) or without (●) 100 μ*M* GTPγS at *p*H 6.0. [Reprinted from (*51*) with permission.]

it has been suggested that cAMP may act by preventing the rise in intracellular Ca^{2+} necessary for a response to stimulation (*40*).

A role for guanine nucleotide binding proteins (G proteins) in the activation of PLC was initially suggested by studies showing that guanine nucleotides reduce the Ca^{2+} requirement for secretion in permeabilized mast cells and platelets (*41*). Litosch *et al.* demonstrated that serotonin promoted the breakdown of phosphoinositides by PLC in membranes from blowfly salivary glands and that guanine nucleotides potentiated this response (*42*). The ability of nonhydrolyzable analogues of guanosine triphosphate (GTP) to stimulate breakdown of endogenous phosphoinositides has now been demonstrated in many systems, including human neutrophils (*43*), hepatocyte membranes (*44*), cerebral cortex membranes (*45*), and GH_3 pituitary cells (*46*). These studies have led to the concept that the control of PLC may occur in a manner analogous to that of adenylate cyclase. Occupancy of receptors coupled to adenylate cyclase leads to formation of a complex between a G protein and the agonist-occupied receptor. After exchange of bound guanosine diphosphate (GDP) for GTP on the G protein, the receptor is released from the complex and the G protein dissociates into its α and βγ subunits (*47*). The α subunit then stimulates [if it comes from a stimulatory G protein (G_s) (*48*)] or inhibits (G_i) (*49*) adenylate cyclase. This effect on adenylate cyclase activity is terminated by GTPase activity intrinsic to the G proteins (*50*). Thus, nonhydrolyzable GTP analogues have a more pronounced effect.

Studies showing guanine nucleotide stimulation of polyphosphoinositide-specific PLC in membranes have led to the concept that there may be membrane-bound PLC enzymes distinct from the more prevalent soluble enzymes. According to this idea, the membrane-bound enzyme is coupled to receptors and initiates phosphoinositide breakdown. It is possible that the membrane PLC enzyme or enzymes may represent the soluble enzyme that is specifically associated with the membrane or is a contaminant in the membrane fraction (*51*). Whether or not distinct membrane PLC enzymes exist awaits their isolation and comparison to the soluble enzymes.

We have recently found an effect of guanine nucleotides on cytosolic PLC activity that may function in regulating PLC (*51*). We used a soluble (high-speed supernatant) fraction from platelets and assayed the PLC activity in this preparation. We used unilamellar vesicles of total platelet lipids as substrates to exclude all membrane protein and detergents while still retaining the natural lipid composition. Vesicles thus constructed from whole platelet lipids are very poor substrates for PLC because they are rich in PC. We labeled the vesicles with trace levels of [^{3}H]PtdIns(4,5)P_2 or [^{32}P]PtdIns and measured PLC activity. Hydrolysis of [^{3}H]PtdIns(4,5)P_2 was markedly stimulated by guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (Fig. 3). The hydrolysis of PtdIns was minimally affected by guanine nucleotides. GTPγS was most potent in stimulating PLC, followed by guanosine 5′-(β,γ-imido)triphosphate (GppNHp), GTP, and finally GDP. Guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) reversed GTPγS-mediated stimulation. The effect of GTPγS on PLC activity is not direct because the nucleotide has no effect on the activity of isolated PLC-I or PLC-II. Our study indicates that soluble phospholipase C enzymes have properties similar to those reported previously for membrane-bound enzyme, that is, guanine nucleotide–dependent preference for polyphosphoinositides.

The finding of GTPγS-stimulated PLC activity in a platelet soluble fraction implies that G proteins may be cytosolic as well as membrane bound. The function of G proteins that modulate adenylate cyclase is affected by the bacterial toxins, cholera toxin and pertussis toxin. These toxins catalyze the adenosine diphosphate (ADP) ribosylation of the α subunits of G_i, G_s, G_o, and transducin (*52*). Cholera toxin–modified G_s persistently activates adenylate cyclase, and pertussis toxin–ADP-ribosylated G_i is unable to inhibit adenylate cyclase activity. Nakamura and Ui (*53*) recently found a cytosolic substrate for pertussis toxin in mast cells, suggesting that G proteins are soluble in some cases. We also find a soluble substrate for pertussis toxin in platelets. Sternweis has recently shown that the dissociated α subunits of brain G_s and G_o are soluble without detergents (*54*). Also, Rodbell demonstrated that G proteins can translocate from one membrane to another (*55*). Lynch *et al.* (*56*) have shown that α subunits of G_s are released from liver plasma membranes after cholera toxin activation. Additionally, Bhat *et al.* (*57*) have shown that a variety of tissues contain a soluble guanine nucleotide–binding protein activity that can reconstitute adenylate cyclase activity in membranes from cyc^- cells that lack G_s. Taken together, these studies show that G proteins or subunits thereof can be soluble. We speculate that a soluble G protein may bind to soluble PLC thereby leading to its association with a membrane. The nature of the G protein that stimulates PLC is unknown. In our soluble PLC and soluble G-protein system, there is a pertussis toxin substrate that has a similar size to $G_{\alpha i}$ by SDS–polyacrylamide gel electrophoresis. Pertussis toxin interferes with phosphoinositide turnover in some tissues but not in others (*58*). We have not yet isolated this soluble G protein nor have we succeeded in reconstituting guanine nucleotide dependence of a homogeneous PLC preparation.

Inositol Phosphates

Inositol cyclic trisphosphate. The inositol phosphate product of PLC hydrolysis of PtdIns is a mixture of Ins 1P and an inositol cyclic phosphate ester [cIns(1:2)P] (Fig. 4). Dawson studied the effects of *p*H on the proportions of cIns(1:2)P and Ins 1P produced by the enzyme in vitro (*59*). At *p*H 4.4, 88% of the product was the cyclic product; at *p*H 6.8, the percentage decreased to 45%. Both Ins 1P and cIns(1:2)P appeared to be released from the substrate simultaneously and no breakdown of the cyclic compound was observed under the conditions of the assay. These observations are consistent with the idea that either the 2-position hydroxyl on the inositol ring or free OH^- in solution may attack the phosphorus. As the *p*H increases, hydrolysis is preferred over formation of a cyclic ester by transfer of phosphate to the 2 hydroxyl on the inositol ring. Therefore PLC produces two products rather than sequentially forming inositol cyclic phosphate as an intermediate with inositol monophosphate as the product (as is the case with ribonuclease). Our finding that PLC-I and PLC-II both utilize all three phos-

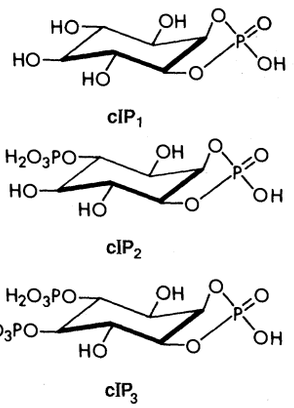
phoinositides as substrates suggests that the enzymes also form cyclic esters from PtdIns4P and PtdIns(4,5)P_2 (Fig. 4). We demonstrated that PLC produces cyclic products from all three phosphoinositide substrates (*60*). Initially, inositol cyclic phosphates were detected by ^{18}O labeling. In the presence of acid, cyclic phosphates are rapidly hydrolyzed to phosphomonoesters and, when the hydrolysis is carried out in $H_2{}^{18}O$, the resultant phosphomonoesters contain ^{18}O. We measured the ^{18}O content of the phosphomonoesters after alkaline phosphatase treatment and conversion of inorganic phosphate to a volatile derivative for gas chromatography and mass spectrometry. We have subsequently isolated the inositol cyclic phosphate products by high-performance liquid chromatography (HPLC) (*61*) and find 60 to 70% cyclic product formed from PtdIns, 40 to 50% from PtdIns4P, and 30 to 40% from PtdIns(4,5)P_2. We have been unable to explain the basis for the differences in proportion of cyclic and noncyclic inositol phosphate products with the various substrates. The proportion of cyclic and noncyclic products is not affected by duration of incubation or amount of enzyme. Both PLC-I and PLC-II produce similar amounts of cyclic and noncyclic products.

The physiological effects of the inositol cyclic phosphates have been examined in several systems. As mentioned above, a number of studies have demonstrated that addition of Ins(1,4,5)P_3 to permeabilized cells results in mobilization of Ca^{2+} from intracellular stores (for reviews, see *1*). Permeabilized platelets release ^{45}Ca in response to Ins(1,4,5)P_3 at micromolar concentrations (*62*). Inositol 1:2-cyclic 4,5-trisphosphate [cIns(1:2,4,5)P_3] is as potent as Ins(1,4,5)P_3 in platelets and in 3T3 cells in this assay, while inositol 1:2-cyclic 4-bisphosphate [cIns(1:2,4)P_2] is inactive (*61*). cIns(1:2,4,5)P_3 was also injected into intact *Limulus* ventral photoreceptors. It elicited a conductance change that resembles the response of the photoreceptor cells to illumination. The cyclic trisphosphate was approximately five times more potent than its noncyclic counterpart in eliciting this response. The basis for this difference in potency may be explained by the slower metabolism of cIns(1:2,4,5)P_3 in cells, as described below. Physiological studies have been hindered by the lack of availability of cIns(1:2,4,5)P_3. We have recently succeeded in synthesizing cIns(1:2,4,5)P_3 from Ins(1,4,5)P_3 with a water-soluble carbodiimide (*63*), which should provide sufficient mass of cIns(1:2,4,5)P_3 to examine these properties in detail.

Several reports indicate that inositol cyclic phosphates are produced in addition to inositol phosphates when cells are stimulated by agonists. cIns(1:2)P has been isolated from pancreas (*64*), kidney (*65*), platelets (*66*), and SV40 transformed mouse cells (*67*). Recently, we isolated cIns(1:2,4,5)P_3 from thrombin-treated platelets (*68*). We find 0.2 to 0.4 nmol per 10^9 platelets at 10 seconds after thrombin treatment; none was found in unstimulated platelets or in platelets 10 minutes after addition of thrombin. Although cIns(1:2,4,5)P_3 is a major product of PtdIns(4,5)P_2 hydrolysis in platelets, in fibrosarcoma cells from the Harlan Sprague-Dawley mouse stimulated with bradykinin much smaller amounts of cIns(1:2,4,5)P_3 have been detected (*69*). Because the inositol phosphates are rapidly metabolized, it is difficult to estimate the amount of a particular compound by measuring its concentration at one or a few time points after stimulation by an agonist.

We have added cIns(1:2,4,5)P_3, cIns(1:2,4)P_2, and cIns(1:2)P to homogenates of platelets and rat kidney to determine the pathway of degradation of these compounds (*70*). We unexpectedly found that cIns(1:2,4,5)P_3 is converted to cIns(1:2,4)P_2 without any apparent conversion to Ins(1,4,5)P_3. Similarly, cIns(1:2,4)P_2 was converted to cIns(1:2)P without any apparent formation of Ins(1,4)P_2. Thus, it appears that the cyclic and noncyclic inositol phosphates are metabolized separately to the monophosphate level, at which point cIns(1:2)P is hydrolyzed to Ins 1P by the cIns(1:2)P hydrolase. This Ins 1P is then hydrolyzed by the Li^+-sensitive phosphatase to yield free inositol and inorganic phosphate (*71*). These pathways are summarized in Fig. 2.

Fig. 4. Structures of inositol cyclic phosphates. cIP_1, cIns(1:2)P; cIP_2, cIns(1:2,4)P_2; cIP_3, cIns(1:2,4,5)P_3.



Inositol 1:2 cyclic phosphate hydrolase. This enzyme and its ubiquitous distribution was first described by Dawson and Clark (*72*). In most tissues, the majority of the enzyme is cytosolic, although some sediments with the particulate fraction. In the presence of Mn^{2+} or Mg^{2+} ions at *p*H 7.8, the enzyme catalyzes the formation of Ins 1P from cIns(1:2)P. We have recently isolated this enzyme from the soluble fraction of human placenta (*73*). The isolated enzyme has an apparent molecular weight of 55,000 as determined by gel filtration chromatography. The molecular weight upon SDS–polyacrylamide gel electrophoresis was 29,000, both in the presence and absence of 2-mercaptoethanol. The enzyme is specific and does not hydrolyze other cyclic phosphate esters, including 2′,3′-cAMP or 3′,5′-cAMP. The cIns(1:2)P hydrolase also does not hydrolyze the cyclic phosphate esters of the inositol cyclic polyphosphates, which is consistent with the findings in crude platelet extracts. The enzyme is strikingly inhibited by inositol 2-phosphate (Ins 2P) (50% inhibition at 4 μ*M*), while its product Ins 1P is much less inhibitory (50% inhibition at 2 m*M*). The physiological significance, if any, of inhibition by Ins 2P is unknown.

Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4. Recent studies indicate that the metabolism of inositol phosphates is considerably more complex than originally proposed. Irvine and co-workers (*74*) isolated the inositol trisphosphates from carbachol-stimulated rat parotid glands (15-minute stimulation) and found that the major inositol trisphosphate was not the expected Ins(1,4,5)P_3 isomer but rather inositol 1,3,4-trisphosphate [Ins(1,3,4)P_3]. This finding was puzzling because there was no corresponding lipid precursor for this moiety, PtdIns(3,4)P_2. The source of this isomer was elucidated by the discovery by Batty, Nahorski, and Irvine (*75*) that rat brain cortical slices stimulated with carbachol rapidly formed inositol 1,3,4,5-

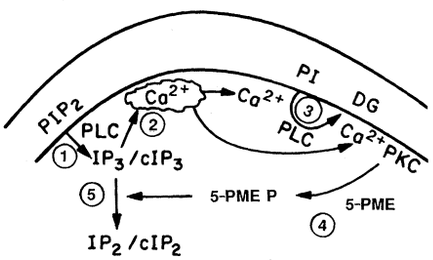


Fig. 5. Pathway of proposed phosphoinositide-induced Ca^{2+} mobilization. (1) Phospholipase C (PLC) cleaves Ins(1,4,5)P_3 (IP_3) and cIns(1:2,4,5)P_3 (cIP_3) from PtdIns(4,5)P_2 (PIP_2). (2) IP_3 and cIP_3 mobilize Ca^{2+}. (3) PLC cleaves PtdIns (PI) to form diacylglyceride (DG) and Ins 1P. (4) DG, Ca^{2+}, and protein kinase C (PKC) phosphorylate IP_3 5-phosphomonoesterase (5-PME). (5) Phosphorylated 5-PME hydrolyzes IP_3 and cIP_3. [Adapted from (*81*) with permission.]

tetraphosphate [Ins(1,3,4,5)P_4]. This suggested the presence of an Ins(1,4,5)P_3 3-phosphokinase that converts Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4. This kinase has been demonstrated in the soluble fraction of homogenates of brain, liver, pancreas, and platelets (*76*). The enzyme appears to have a very high affinity for Ins(1,4,5)P_3 (The K_m is approximately 0.6 μM), much higher than the competing Ins(1,4,5)P_3 5-phosphomonoesterase described below. The tetraphosphate product of the kinase reaction is a substrate for inositol trisphosphate 5-phosphomonoesterase (5-PME), which converts it to Ins(1,3,4)P_3, thereby explaining the presence of this isomer in stimulated tissues. Thus, a second pathway for Ins(1,4,5)P_3 metabolism can be written as:

$$\text{Ins(1,4,5)P}_3 \xrightarrow[\text{kinase}]{\text{ATP}} \text{Ins(1,3,4,5)P}_4 \xrightarrow[\text{5-PME}]{} \text{Ins(1,3,4)P}_3$$

In many tissues Ins(1,3,4)P_3 appears very rapidly after agonist stimulation, implying that kinase and 5-phosphomonoesterase rapidly metabolize Ins(1,4,5)P_3 via this pathway (*74–77*). Whether Ins(1,3,4,5)P_4 or Ins(1,3,4)P_3 are themselves distinct messenger molecules is uncertain because there have been no reports of physiological experiments with these compounds. The further metabolism of Ins(1,3,4)P_3 has not been fully elucidated, although there are reports of 1-phosphatase, 4-phosphatase, and 3-phosphatase enzymes that are likely to participate in its further metabolism (*76*). Thus Ins(3,4)P_2, Ins(1,3)P_2, or Ins(1,4)P_2, respectively, could be formed. Further studies on the enzymes and the products formed will clarify this pathway.

Ins(1,4,5)P_3 5-phosphomonoesterase. The generation of inositol trisphosphates by PLC cleavage of PtdIns(4,5)P_2 may be considered analogous to the adenylate cyclase system. Thus, the PLC enzyme is analogous to adenylate cyclase, and in this case produces Ins(1,4,5)P_3 and cIns(1:2,4,5)P_3 as second messengers, just as adenylate cyclase produces cAMP. The Ins(1,4,5)P_3 5-phosphomonoesterase degrades both of these inositol trisphosphates to inert (at least with respect to Ca^{2+} mobilization) inositol phosphates, Ins(1,4)P_2, and cIns(1:2,4)P_2, respectively. Thus, the Ins(1,4,5)P_3 5-phosphomonoesterase is analogous to the cyclic nucleotide phosphodiesterase and has the potential to control the amount of inositol phosphate messenger molecules, as does PLC.

The 5-phosphomonoesterase has been described in erythrocytes, blowfly salivary glands, brain, smooth muscle, pancreatic islets, and liver (*78*). In these tissues, the activity appears to be mainly in the particulate fraction, although in platelets we find that the enzyme is mostly soluble (*79*). We purified the enzyme to homogeneity from platelets. The isolated enzyme is a single polypeptide that has an apparent molecular weight of about 45,000 by SDS–polyacrylamide gel electrophoresis. We originally reported that the enzyme had a molecular weight of 38,000; however, in subsequent studies we find that the enzyme migrates on SDS–polyacrylamide gel electrophoresis just above actin, which has a molecular weight of approximately 45,000. The enzyme requires Mg^{2+} and is active in crude extracts of most tissues, where it has the capacity to degrade within a few seconds any Ins(1,4,5)P_3 that is formed. The enzyme is specific for Ins(1,4,5)P_3 (*78, 79*) and Ins(1,3,4,5)P_4; it removes the phosphate in position 5 from either substrate. The enzyme does not hydrolyze Ins(1,4)P_2 or glycerophosphorylinositol derivatives (GroPIns), such as glycerophosphorylinositol (4,5)-bisphosphate [GroPIns(4,5)P_2] or glycerophosphorylinositol 4-phosphate (GroPIns4P) (*79*). The enzyme does hydrolyze the lipid substrate, PtdIns(4,5)P_2, at approximately 0.01% of the rate at which it hydrolyzes Ins(1,4,5)P_3.

The catalytic efficiency of the Ins(1,4,5)P_3 5-phosphomonoesterase in hydrolyzing Ins(1,3,4,5)P_4 is much less than that observed with Ins(1,4,5)P_3 (*80*). The maximal velocity of hydrolysis of the Ins(1,3,4,5)P_4 substrate is approximately one-thirtieth of that observed with Ins(1,4,5)P_3. However, the affinity for Ins(1,3,4,5)P_4 is greater than that for Ins(1,4,5)P_3; the K_m value is approximately 10% of that observed for the Ins(1,4,5)P_3 substrate. These parameters suggest that at low substrate concentrations, Ins(1,3,4,5)P_4 is hydrolyzed rapidly, but at higher substrate concentrations the capacity to cleave Ins(1,4,5)P_3 is much greater. The platelet 5-phosphomonoesterase also hydrolyzes cIns(1:2,4,5)P_3, although the capacity of the enzyme to utilize this substrate is less than 10% of its ability to hydrolyze Ins(1,4,5)P_3 at substrate concentrations that might occur in a stimulated cell (*70*). This, plus the finding that Ins(1,4,5)P_3 3-phosphokinase does not utilize cIns(1:2,4,5)P_3 as a substrate (*80*), suggests that the cyclic inositol trisphosphate may be more stable in cells than its noncyclic counterpart. Therefore, even if the amount of the cyclic trisphosphate produced is small, it may have a more prolonged effect than the noncyclic trisphosphate.

We recently discovered that the Ins(1,4,5)P_3 5-phosphomonoesterase enzyme is phosphorylated by protein kinase C (*81*). The enzyme appears to be phosphorylated on multiple sites with approximately 3 to 4 mol of phosphate per mole of enzyme. Phosphorylation of the enzyme by protein kinase C results in activation of the phosphatase enzyme. We find two- to tenfold stimulation of Ins(1,4,5)P_3 5-phosphomonoesterase activity using various preparations of the phosphatase. Presumably, the variation in the degree of stimulation results from variable degrees of "basal" phosphorylation of the enzyme used in the phosphorylation reaction. The extent of phosphorylation of the Ins(1,4,5)P_3 5-phosphomonoesterase in the cell remains to be determined. We have not yet succeeded in dephosphorylating the enzyme to determine the intrinsic activity of the unphosphorylated protein. The phosphorylation of the phosphatase increases its activity approximately equally toward all three of its known substrates—Ins(1,4,5)P_3, cIns(1:2,4,5)P_3, and Ins(1,3,4,5)P_4. These findings can explain reports that indicate that treatment of platelets and other cells with phorbol esters (compounds that activate protein kinase C) decreases the subsequent rise in Ca^{2+} in response to agonists, decreases the levels of Ins(1,4,5)P_3, and causes rapid degradation of Ins(1,4,5)P_3 added to permeabilized cells (*82*).

Over 10 years ago we discovered that thrombin stimulation of intact $^{32}PO_4$-labeled platelets was associated with the rapid increase in the incorporation of phosphate into proteins with molecular weights of 20,000 (20K) and 40,000 (40K) (*83*). The former is the light chain of myosin (*84*), and the latter is a major substrate for protein kinase C in stimulated platelets (*85*). We have now shown that the phosphorylated 40K protein from thrombin-stimulated platelets comigrates on SDS–polyacrylamide gel electrophoresis with Ins(1,4,5)P_3 5-phosphomonoesterase phosphorylated with protein kinase C. *Staphylococcus aureus* V8 protease and tryptic peptide maps of the two proteins are identical. Thus the 40K protein that is phosphorylated in platelets in response to thrombin appears to be the Ins(1,4,5)P_3 5-phosphomonoesterase. Although this protein is reported as a 40K protein (*83, 85*), its actual molecular weight is 45,000, as mentioned above.

Our hypothesis for the regulation of Ins(1,4,5)P_3 production and phosphoinositide-induced Ca^{2+} mobilization by the 5-phosphomonoesterase is shown in Fig. 5. Thrombin stimulation of platelets activates phospholipase C (PLC) to cleave PtdIns(4,5)P_2 (PIP_2) to form Ins(1,4,5)P_3 (IP_3), cIns(1:2,4,5)P_3 (cIP_3), and 1,2-diacylglycerol (not shown). These inositol phosphates mobilize Ca^{2+} from intracellular stores to elevate the cytosolic Ca^{2+} concentration, which allows phospholipase C to utilize PtdIns (PI) as a substrate to form Ins 1P, cIns(1:2)P, and 1,2-diacylglycerol (DG). The 1,2-diacylglycerol derived from PtdIns and PtdIns(4,5)P_2 plus the elevated cytosolic Ca^{2+} activate protein kinase C, which phosphorylates the 5-phosphomonoesterase (5-PME) and thereby activates the

phosphatase. Finally, the phosphorylated 5-phosphomonoesterase (5-PME P) hydrolyzes Ins(1,4,5)P_3 and cIns(1:2,4,5)P_3. The Ins(1,4)P_2 (IP_2) and cIns(1:2,4)P_2 (cIP_2) formed are inactive, and therefore, the phosphoinositide-derived signal is terminated.

Role of Phosphoinositide Turnover in Cell Growth

There is considerable interest in the role that phosphatidylinositol turnover plays in regulating cell growth. A relationship between phosphoinositides and cell growth is suggested by the finding that several growth factors, including platelet-derived growth factor and epidermal growth factor, stimulate phosphoinositide turnover (*86*). In addition, the tumor-promoting phorbol esters stimulate protein kinase C directly, possibly bypassing the phosphoinositide-derived diacylglycerol cofactor. Thus alterations in the phosphoinositide signal pathway could affect the control of cell growth. The stimulation of phosphoinositide turnover in the transforming process of a tumor virus was first shown in studies of Rous sarcoma virus transformed quail cells (*87*). More recently, it was suggested that the transforming protein of tumor viruses that are protein tyrosine kinases are also lipid kinases that can convert PtdIns to PtdIns4P (*88*). A difficulty with the hypothesis was that the PtdIns kinase activity of these protein tyrosine kinases was meager compared to cellular PtdIns kinase. Further studies have refuted the hypothesis by showing that the transforming protein tyrosine kinases are devoid of PtdIns kinase activity (*89*). However, these studies confirm the finding that phosphoinositide turnover is increased in transformed cells. Furthermore, membranes from V-*fms* and V-*fes* transformed cells have increased PtdIns(4,5)P_2-specific PLC activity compared to uninfected cells (*90*). Thus there remains a possibility that derangements of the phosphoinositide messenger system may be important in the development of uncontrolled growth in transformed cells.

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Research Articles

Structure of the DNA–Eco RI Endonuclease Recognition Complex at 3 Å Resolution

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The crystal structure of the complex between Eco RI endonuclease and the cognate oligonucleotide TCGCGAATTCGCG provides a detailed example of the structural basis of sequence-specific DNA-protein interactions. The structure was determined, to 3 Å resolution, by the ISIR (iterative single isomorphous replacement) method with a platinum isomorphous derivative. The complex has twofold symmetry. Each subunit of the endonuclease is organized into an α/β domain consisting a five-stranded β sheet, α helices, and an extension, called the "arm," which wraps around the DNA. The large β sheet consists of antiparallel and parallel motifs that form the foundations for the loops and α helices responsible for DNA strand scission and sequence-specific recognition, respectively. The DNA cleavage site is located in a cleft that binds the DNA backbone in the vicinity of the scissile bond. Sequence specificity is mediated by 12 hydrogen bonds originating from α helical recognition modules. Arg^{200} forms two hydrogen bonds with guanine while Glu^{144} and Arg^{145} form four hydrogen bonds to adjacent adenine residues. These interactions discriminate the Eco RI hexanucleotide GAATTC from all other hexanucleotides because any base substitution would require rupture of at least one of these hydrogen bonds.

THE ABILITY OF A PROTEIN TO RECOGNIZE A SPECIFIC sequence of bases along a strand of double helical DNA lies at the heart of many fundamental biological processes. One of the most intriguing questions in molecular biology today is whether the details of these individual recognition mechanisms will form a small number of simple patterns that would lead to the development of a general recognition code.

This interest has stimulated crystallographic studies on many proteins that recognize specific sequences of DNA. The structures of four of these have been solved in the absence of DNA; these proteins are the Cro and CI repressors from coliphage λ, the *Escherichia coli* catabolite gene activator protein (CAP) and the tryptophan repressor (*1–6*). These four proteins share a common "helix-turn-helix motif" at the suggested DNA binding site, which has led to model building of the recognition complexes (*7–9*). In addition, the 7 Å structure of a co-crystalline complex between coliphage 434 repressor and a tetradecanucleotide containing its specific operator sequence supports the general features of these

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