Type I Phosphatidylinositol 4-Phosphate 5-Kinase Isoforms Are Specifically Stimulated by Phosphatidic Acid*

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A phosphatidylinositol 4-phosphate (PIP) kinase was isolated and purified to near homogeneity from bovine erythrocyte membranes. The PIP kinase was extracted from bovine erythrocyte membranes with a high salt wash, followed by phosphocellulose and phenyl-Sepharose chromatography. The predominant protein after phenyl-Sepharose purification had a molecular size of 68 kDa. Renaturation of PIP kinase activity after SDS-PAGE showed that a 68-kDa protein was able to phosphorylate PIP. An antibody developed against the 38-kDa protein Western blots the 68-kDa protein and is able to immunoprecipitate the 68-kDa protein and PIP kinase activity from membrane extracts. Based on functional studies, the 68-kDa protein is indistinguishable from the type I PIP kinase previously characterized from human erythrocyte membranes (Bazenet, C. E., Ruano, A. R., Brockman, J. L., and Anderson, R. A. (1990) J. Biol. Chem. 265, 18012-18022). These studies also show that the type I PIP kinases, but not the type II PIP kinase, are stimulated by phosphatidic acid, suggesting alternative roles for these enzymes. Two immunoreactive isoforms of the type I PIP kinase, of 68 and 90 kDa, were identified in rat brain and partially purified. Both of these isoforms are also stimulated by phosphatidic acid.

The second messengers, inositol 1,4,5-trisphosphate and 1,2-diaclyglycerol, are produced upon agonist activation of phospholipase C. Inositol 1,4,5-trisphosphate increases the intracellular concentration of calcium, and 1,2-diaclyglycerol stimulates the activity of protein kinase C (for reviews, see Refs. 2 and 3). The production of these second messengers is dependent on the hydrolysis of phosphatidylinositol 4,5-bisphosphate. Phosphatidylinositol 4,5-bisphosphate (PIP₂) is produced by the sequential phosphorylation of phosphatidylinositol by phosphatidylinositol 4-kinase and phosphatidylinositol 4-phosphate 5-kinase. Both of these enzymes have been purified from a variety of sources (for a review, see Ref. 3). In human erythrocytes, there appears to be a single phosphatidylinositol kinase (3, 4), while there are two distinct PIP kinases, termed type I and type II (1, 5). The type II PIP kinase has been purified to homogeneity and shown to be structurally and functionally distinct from the type I PIP kinase by kinetic studies, specificity in use of GTP and ATP, the effects of modulators, and immunoreactivity (1). More recently, multiple PIP kinases have been characterized in bovine brain preparations (6–9). Divecha and co-workers (9) have evidence suggesting that there are at least three distinct brain PIP kinases, termed type A, B, and C. Based on cross-reactivity of the antibodies and kinetic studies, the type C PIP 5-kinase is the brain isoform of the type II PIP 5-kinase originally isolated from human erythrocytes. The erythroid and brain type II (or C) PIP 5-kinases have identical sizes and immunoreactivity (9).

The polyphosphoinositide kinases are reportedly regulated by small G-proteins, tyrosine phosphorylation, Ca²⁺-calmodulin, phosphatidic acid, and polyamines (3, 10–13). In general, activity is stimulated by Mg²⁺ ions and phosphatidylserine and is inhibited by the product, PIP₂. The type II PIP 5-kinase is inhibited by beparin, whereas the type I is stimulated. The type I PIP 5-kinase is stimulated by spermine, while type II is inhibited or not affected, depending upon the assay conditions (1). Another potential modulator is GTPγS, which has been reported to stimulate PIP kinase activity in rat brain and placental membranes (10–12).

More recently, Moritz and co-workers (13) have demonstrated that PA could stimulate the activity of a PIP kinase isolated from bovine brain. Furthermore, PIP₂ production is increased in isolated rat brain synaptosomes upon treatment with phospholipase D, an enzyme that stimulates PA formation (14). This was an important observation since it suggests a mechanism for the regulation of PIP 5-kinases in response to PIP₂ breakdown and subsequent production of PA. Since there are multiple PIP kinases present in the brain (9): a subset of these PIP kinases may be stimulated by PA. Moreover, in vivo and in vitro stimulation by PA may be indirect and may act on components that regulate the PIP kinases. To address these questions, a type I PIP kinase has been isolated and an antibody has been prepared that is specific for this enzyme. The erythroid type I PIP 5-kinase is shown to be directly stimulated by PA and two brain homologs of type I are identified that are also stimulated by PA. These are distinct from the erythroid and brain type II PIP 5-kinases, which are not stimulated by PA.

EXPERIMENTAL PROCEDURES

Materials—Whatman P-11 phosphocellulose and silica gel 60 TLC plates were purchased from Whatman. [γ³²P]ATP was purchased from DuPont NEN. Q-Sepharose and the Mono-Q column were obtained from Pharmacia LKB Biotechnology Inc. PIP was prepared as described (1). All other phospholipids were from Avanti Polar Lipids. All other chemicals were bought from Sigma or were of reagent grade.

Preparation of Bovine Erythrocyte Membranes—Approximately 4 li-

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‡ The abbreviations used are: PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PIP 5-kinase, phosphatidylinositol 4-phosphate 5-kinase; PI, phosphatidylinositol; PA, phosphatidic acid; PBS, phosphate-buffered saline; PMSE, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PLC, phospholipase C; GTPγS, guanosine 5'-O-(thiotriphosphate).
were macerated with a plastic pestle and incubated for 18 h on a tube tumbler with 1.5 ml of elution buffer. After incubation, the acrylamide gel slices of 5 mm were excised and washed extensively with PBS. Approximately 150 pg of the eluted 68-kDa protein was mixed with Freund's complete adjuvant (1:1) and injected subcutaneously at multiple sites in 8-week-old female New Zealand White rabbits. The rabbits were boosted after 5 weeks with 50 µg of protein in Freund's incomplete adjuvant (1:1). One antibody production was detected, the rabbits were injected intravenously bimonthly with 50 µg of the electroeluted 68-kDa protein.

All antibodies were affinity-purified on a Sepharose-4B column, which was coupled with the 68-kDa SDS-PAGE-purified protein. Approximately 2 mg of the purified 68-kDa protein was coupled to 8 ml of CNBr-activated Sepharose-4B as described (1, 16). Antibodies were purified by applying 40 ml of antisera, pooled from two rabbits, to the column. The column was washed with PBS containing 500 mM NaCl until the optical density at 280 nm was below 0.05. Then, the antibodies were eluted with 0.1 M glycine at pH 2.5. The eluate was neutralized and dialyzed against PBS.

Purification of a 68-kDa PIP Kinase—Phosphatidylinositol 4-phosphate (PIP) kinase activity was extracted from bovine erythrocyte membranes by a 1 M NaCl wash. This salt concentration removed the majority of detectable PIP kinase activity from the bovine erythrocyte membranes, similar to previous results with human erythrocytes (1). The extract was dialyzed against a phosphate buffer and eluted with a 5-M NaCl gradient. The peak of PIP kinase activity was eluted at salt concentrations identical to those of the human erythrocyte type I PIP kinase (1); however, unlike the human erythrocyte kinase, the bovine erythrocyte PIP kinase activity correlated well with the elution of a 68-kDa protein. Like the human preparation, a 53-kDa protein with PIP kinase activity and immunoactivity with the human 53-kDa type II PIP kinase antibody eluted at 1 M NaCl and was pure (1).

The phosphocellulose column gave a 61-fold purification of the first peak of PIP kinase activity relative to the membrane extract (Table I). The activity peak from this column was placed...
directly on a phenyl-Sepharose column and washed with low ionic strength buffer. The PIP 5-kinase activity was tightly retained by the phenyl-Sepharose and required detergent for elution. Fig. 1 shows the protein and PIP 5-kinase activity elution profile with a 0–0.7% Nonidet P-40 detergent gradient. To remove the detergent and concentrate the PIP kinase, fractions containing PIP kinase activity were applied to a Q-Sepharose column, extensively washed, and eluted with a 800 mM step gradient of NaCl. The peak of PIP kinase activity was pooled, dialyzed into buffered glycerol, as described under "Experimental Procedures," and stored at –20 °C. Under these conditions, no appreciable decrease in kinase activity was detected after several months of storage.

The recovery and specific activity of the enzyme at each step of the purification is summarized in Table I. The two steps described yielded nearly 65-fold purification of the enzyme from the high salt membrane extract. The purified PIP kinase was shown to be nearly homogeneous by silver-stained SDS-PAGE. Numerous other column preparations, a lower molecular mass protein of 53 kDa was observed, which also correlates with activity.

Renaturation of PIP Kinase Activity—In order to clearly identify the catalytic subunit of the PIP kinase, the peak of activity from the phosphocellulose column was resolved by SDS-PAGE, and the gel was cut into 5-mm slices. Adjacent lanes were loaded with molecular weight markers and a smaller amount of protein for silver staining. The excised slices were homogenized, the protein was eluted, and PIP kinase activity renatured according to the methods outlined under "Experimental Procedures." As shown in Fig. 2, the kinase activity was renatured in slices containing the 68- and 53-kDa proteins. In all preparations of PIP kinase, only gel slices that contained the 68- and 53-kDa protein bands had PIP kinase activity. Other contaminating proteins did not have detectable kinase activity.

Preparation of Polyclonal Antibodies to the Putative 68-kDa PIP Kinase—To further characterize the 68-kDa protein, the proteins in a peak of kinase activity from the phosphocellulose elution were purified by preparative SDS-PAGE. The 68-kDa protein was electroeluted from the gel and used to raise polyclonal antibodies in rabbits. Antibodies were affinity-purified from the antiserum using the 68-kDa protein cross-linked to CNBr-activated Sepharose-4B. The resulting antibody specifically recognizes the 68-kDa protein by Western blotting (Fig. 5). The antibody raised to the 68-kDa protein and affinity-purified on the 68-kDa protein-Sepharose column also recognizes the 53-kDa protein. Since the 53-kDa protein was neither consistently present in kinase preparations nor detected by Western blotting of red cell membranes, the 53-kDa band may be a proteolytic fragment of the 68-kDa PIP kinase which retains kinase activity.

Immunoprecipitation of Type I PIP Kinase Activity—The antibody specific for the 68-kDa protein was able to immunoprecipitate (deplete) PIP kinase activity from the high salt membrane extract (Fig. 3) and from the phosphocellulose column peak of activity (not shown). When the supernatant and pellet of the immunoprecipitates were Western blotted with the 68-kDa protein antibody, a 68-kDa protein was detected whose amount increased in a dose-dependent manner concurrent with an increase in PIP kinase activity in the pellet (Fig. 3). Kinase activity in the pellet begins to decrease at higher concentrations of antibody, even though the 68-kDa band is still precipitated. This may be due to antibody inhibition of kinase activity. When the 68-kDa protein was immunoprecipitated from the peak of activity eluted from the phosphocellulose column, this depleted the PIP kinase activity and the 68-kDa protein from these fractions.

To demonstrate that the 68-kDa protein is the type I PIP kinase, we have Western blotted a phosphocellulose elution profile from both human and bovine preparations using the 68-kDa antibody. In Fig. 4, the elution from phosphocellulose of the type I PIP 5-kinase extracted from human erythrocyte membranes is shown. The corresponding Western blot showed that the 68-kDa protein eluted identically with PIP kinase activity. When the 125I-protein A-labeled 68-kDa band was

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**Fig. 1.** Phenyl-Sepharose purification of erythroid type I PIP kinase. The 1 ml extract from bovine erythrocyte membranes was applied to a 30-ml phosphocellulose column and eluted with a 300–1000 mM linear NaCl gradient. PIP kinase activity and the OD280 were measured. The first and largest peak of PIP kinase activity eluted at 0.6–0.7 M NaCl, as with the human kinases (1). This activity peak was applied directly to a 1-ml phenyl-Sepharose column, washed with low ionic strength buffer, and eluted with a 0–0.07% (w/v) Nonidet P-40 gradient. The protein elution was monitored by assaying activity, SDS-PAGE, and silver staining.
Experimental Procedures. PIP kinase activity was determined following renaturation (right panel; proteins were eluted from the slices and renatured, as described under "Experimental Procedures." PIP kinase activity was determined following renaturation (right panel). Fractions were assayed for activity and compared to the Western blot. The autoradiogram of the Western blot was quantitated by densitometric laser scanning (open circles) and is plotted along with the human type I PIP kinase activity (closed circles). This same experiment was done with the bovine PIP kinase elution from phosphocellulose with identical results (not shown).

**Fig. 2. Renaturation of PIP kinase after SDS-PAGE.** The peak of PIP kinase activity from the phosphocellulose column was resolved by SDS-PAGE on a 7–15% gel. Half of the gel was stained with silver (left panel), and the other half of the gel was sliced into 5-mm sections. The proteins were eluted from the slices and renatured, as described under "Experimental Procedures." PIP kinase activity was determined following renaturation (right panel).

**Fig. 3. Immunoprecipitation of PIP kinase activity.** Approximately 50 μg of protein from the high salt extract was incubated overnight with increasing amounts of the affinity-purified 68-kDa protein antibody, in duplicate. Protein A-coupled Sepharose was added, and the immunoprecipitates were collected following a 3-h incubation. In panel A, the supernatants (S) and pellets (P) were subjected to SDS-PAGE and Western blotted with the 68-kDa PIP kinase antibody. The pellets were also assayed for PIP kinase activity (panel B). In experiments in which the 68-kDa protein was immunoprecipitated from the phosphocellulose peak of activity, over 85% of the kinase activity was removed. PIP kinase activity is quantitated, this also corresponded with PIP kinase activity (Fig. 4). This indicated that the 68-kDa protein is the type I PIP kinase that was previously shown to be a PIP 5-kinase (1).

The 68-kDa type I PIP 5-kinase in some preparations appears to be proteolized to a 53-kDa protein (see Figs. 1 and 2) that is the same size as the type II PIP 5-kinase (1). Previously, peptide mapping was done on this 53-kDa protein, which corresponded to type I PIP 5-kinase activity. When the peptide map was compared to the type II PIP 5-kinase peptide map, the proteins were distinct (1). To demonstrate that the 68-kDa type I and 53-kDa type II PIP 5-kinases are immunologically distinct, the purified enzymes from human and bovine erythrocytes were Western blotted with both type I and type II PIP kinase antibodies. As shown in Fig. 5, the type I antibody detected only the 68-kDa protein and the type II antibody detected only the 53-kDa protein. This strongly suggests that these enzymes have dissimilar sequences.

Effect of PA on PIP Kinase Activity—Previously, Moritz et al. (13) have shown that a PIP 5-kinase is potently stimulated by PA, both in vitro and in intact synaptosomes. To determine whether this important regulatory feature is intrinsic to type I or II PIP 5-kinases (or other isoforms), the effect of a variety of phospholipids on the type I and II PIP kinase activities was assessed. As shown in Fig. 6, the only phospholipid assayed that had a large effect on PIP kinase activity was PA. In this assay, PA was potently stimulatory toward type I PIP kinase but had little effect on the type II PIP kinase.

The effect of PA on PIP kinase activity was further characterized in several ways. First, the PIP kinase stimulation was evaluated using the purified type I or II PIP kinases. Under these conditions, the bovine and human erythroid type I PIP kinase shows an 8–15-fold increase in activity in the presence of 80 μM PA, although in some assays, activity was stimulated up to 50-fold (Fig. 6). Type I PIP kinase activity is stimulated both in the presence and absence of detergent, but in the ab-
After SDS-PAGE and Western blotted with either the type I antibody or type II antibody. The pellets were assayed for kinase activity in the presence or absence of detergent, the stimulation was only 3-fold under these assay conditions. In Fig. 7, the effect of PA was assayed after the type I PIP kinase was immunoprecipitated with the 68-kDa antibody. The pellets were assayed for kinase activity in the presence or absence of 80 μM PA, as described under “Experimental Procedures.” From left to right, purified bovine erythrocyte type I PIP kinase was stimulated with PA. The bovine 68-kDa type I PIP kinase was immunoprecipitated, washed extensively with PBS, and then assayed with or without PA. The bovine type I PIP 5-kinase from phosphocellulose was separated on SDS-PAGE and renatured with or without PA. The phosphocellulose peak of human type I and type II were assayed in the presence and absence of PA. All assays were done in duplicate; the range of duplicates is shown except for the renaturation, which was replicated multiple times with comparable results.

**Fig. 5. Western blotting of bovine and human PIP kinases.** After phosphocellulose chromatography, approximately equal activities of human (lanes 1 and bovine (lanes 2) type II PIP 5-kinase and human (lanes 3) and bovine (lanes 4) type I PIP 5-kinase were resolved by SDS-PAGE and Western blotted with either the type I antibody (left panel) or type II antibody (right panel). The bound antibodies were detected with 125I-labeled protein A.

**Fig. 6. Effect of different phospholipids on type I PIP kinase activity.** The bovine erythrocyte type I PIP 5-kinase from the phosphocellulose pool was assayed in duplicate in the presence of 0.1% Triton X-100 and 80 μM of the indicated phospholipid using the assay conditions described under “Experimental Procedures.” PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylinositol; PG, phosphatidylglycerol; PI, phosphatidylinositol.

**Fig. 7. Evidence for direct phosphatidic acid stimulation of the 68-kDa type I PIP kinase.** PIP kinase activity from bovine and human erythrocyte preparations was determined in either the presence (solid bars) or absence (hatched bars) of 80 μM PA, as described under “Experimental Procedures.” From left to right, purified bovine erythrocyte type I PIP kinase was stimulated with PA. The bovine 68-kDa type I PIP kinase was immunoprecipitated, washed extensively with PBS, and then assayed with or without PA. The bovine type I PIP 5-kinase from phosphocellulose was separated on SDS-PAGE and renatured with or without PA. The phosphocellulose peak of human type I and type II were assayed in the presence and absence of PA. All assays were done in duplicate; the range of duplicates is shown except for the renaturation, which was replicated multiple times with comparable results.

**Fig. 8. Effect of PA on PIP kinase activity.** The PIP kinase activity using purified bovine erythrocyte type I (open squares) and human erythrocyte type II PIP 5-kinase (closed squares) was assayed with increasing PA in the presence of 0.1% Triton X-100, as described under “Experimental Procedures.” The double-reciprocal plot of the type I PIP kinase stimulation by PA is given (inset). The range of duplicates is shown except when they fall within the symbols.

shown that a PIP kinase activity in synaptosomes is stimulated by PA both in vivo and in vitro. To determine if these PIP kinases are related to the type I or II PIP 5-kinase from erythrocytes, we have Western blotted isolated synaptosomes and brain homogenates (Fig. 9). These results show that bovine brain synaptosomes contain at least two type I PIP kinase immunoreactive proteins with molecular masses of 68 and 90 kDa and a type II PIP kinase immunoreactive protein of 53 kDa.

To determine if these proteins correspond to PIP kinases, brain cytosol was fractionated by phosphocellulose chromatography. Both the 88- and 90-kDa immunoreactive proteins were
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**DISCUSSION**

This paper describes the purification and characterization of the type I PIP kinase from bovine erythrocytes. We have purified the 68-kDa type I PIP 5-kinase to near homogeneity. To clearly demonstrate that the 68-kDa protein is the catalytic subunit, the kinase activity was renatured after SDS-PAGE. The activity was recovered from a 68-kDa protein and, to a lesser extent, from a 53-kDa protein. A polyclonal antibody was raised to the 68-kDa protein and affinity-purified on a 68-kDa protein-Sepharose-4B affinity column. This antibody recognizes the 68- and 53-kDa proteins by Western blotting. The content of 53-kDa protein in the preparations was variable, suggesting that this protein may be a proteolytic product. The antibody raised to the 68-kDa protein immunoprecipitates and immunodepletes PIP kinase activity. Taken together, these results demonstrate that the 68-kDa protein is a PIP kinase.

The type I PIP 5-kinase had been partially purified and characterized from human erythrocytes (1). When the peak of human erythroid type I PIP 5-kinase from the phosphocellulose column was Western blotted with the 68-kDa protein antibody, only a 68-kDa protein was detected, and this protein corresponded to the elution of type I PIP 5-kinase activity from the phosphocellulose column. Since the bovine erythrocyte 68-kDa protein-Sepharose-4B affinity column.
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PIP kinase has properties indistinguishable from the human erythrocyte type I PIP 5-kinase, it suggests that the 68-kDa PIP kinase is the type I PIP 5-kinase. The type I PIP 5-kinases are distinct from the type II PIP 5-kinase previously isolated from human (and bovine) erythrocytes based on its structure and kinetic properties. Moreover, the antibodies raised against the type I kinase do not detect the type II kinase, and the type II kinase antibodies do not detect the type I kinase.

Both the type I and II PIP 5-kinases are present in bovine erythrocytes and brain and many cell types. In bovine brain, there appear to be at least three PIP kinases, termed type A, B, and C (9). The type C PIP kinase corresponds to the erythroid type II kinase based on kinetic and immunological data (9). A partial purification of the bovine brain PIP kinases identified two peaks of kinase activity eluting from a Mono-Q column. These peaks have immunoreactivity with the erythroid type I PIP 5-kinase antibodies. These two bands, termed type Ia and Ib, appear to be the type A and B PIP kinases previously characterized (9). The difference in size between the type Ia and Ib PIP kinases appears not to result from proteolysis since Western blotting of bovine brain synaptosomes homogenized in hot SDS sample buffer showed the same immunoreactive bands. Furthermore, the type Ia PIP kinase can be quantitatively immunoprecipitated from the phosphocellulose peak of activity using the affinity-purified 68-kDa protein antibody. The type Ib PIP kinase is resistant to immunoprecipitation under these conditions. This suggests that the type Ia and Ib are different isoforms. These results confirm that there are at least three PIP kinase isoforms in brain.

Previously, Moritz et al. (13) showed that phosphatidic acid potently stimulated the PIP kinase activity of a purified brain PIP 5-kinase. Moreover, when isolated intact synaptosomes were incubated with phospholipase D, this increased the PA content of the synaptosomes and also stimulated PIP2 production (13). These experiments suggest that PA can stimulate brain PIP kinases both in vitro and in vivo. Results presented in this paper show that the type I PIP kinase is specifically stimulated by PA (Fig. 6) and that both brain type Ia and type Ib PIP kinase isoforms are also stimulated (Fig. 10). The type II kinase from brain and erythrocytes, however, showed no increase in activity. The presence of PA (Figs. 7 and 8). When the type I PIP kinase activity was reassayed following SDS-PAGE or immunoprecipitation, kinase activity was also stimulated by phosphatidic acid. These results suggest that PA directly stimulates type I PIP 5-kinase activity and is not acting through another protein unless this protein has the same electrophoretic mobility as the PIP kinase. Furthermore, since PA did not stimulate the type II kinase activity, it suggests that the mechanism for phosphatidic acid stimulation is unique to the type I PIP 5-kinase isoforms.

The selective regulation of the type I PIP 5-kinases by PA suggests an important regulatory feedback mechanism, which could stimulate the production of PIP2 and the resulting downstream second messengers. Since phosphatidic acid is produced in many cells upon stimulation by different agonists, the possibility of the type I PIP kinases being regulated by PA exists (20-23). PA has been shown to be involved in a variety of cellular events including DNA synthesis, cell growth, and more recently membrane trafficking. Brown et al. (24) showed that ADP-ribosylation factor stimulates phospholipase D activity and thus increases phosphatidic acid levels. Maximum stimulation by ADP-ribosylation factor required PIP2, but not other negatively charged phospholipids, suggesting that PIP kinase activity may be an important regulatory enzyme in membrane trafficking. The observations that PA increased phospholipase C (PLC) activity in vitro (25) and that PLC-1 after tyrosine phosphorylation by the epidermal growth factor receptor was allosterically stimulated by PA (26) suggest that both PIP 5-kinase and PLC activity are feedback stimulated by PA. The PA stimulation may couple the PIP2 generation to the cleavage of PIP2 by activated PLC. There is also evidence that the epidermal growth factor receptor interacts with a PIP 5-kinase (27). The above mechanism is consistent with recent results demonstrating that the PI transfer protein is essential for second messenger generation by PLC (28). Taken together, these results suggest that activation of phosphoinositide-generated second messengers results in a flow of PI from sites of synthesis to sites of PLC activity. The PLC generates diacylglycerol, which is phosphorylated to produce phosphatidic acid. Thus, phosphatidic acid, the end product of PI second messenger production, may stimulate further PI generation and cleavage by PLC.

Immunoreactive proteins of the same size as the erythroid type I and II PIP 5-kinases were detected by Western blotting with the antibodies in all tissues and cells assayed. This suggested that both kinase isoforms are widely distributed. Since the type I and II PIP kinase isoforms are regulated differently, it implies that multiple regulatory pathways are required for production of PIP. Previous reports have shown various subcellular localizations for the PIP kinases (1, 6-10, 29-33). Most characterizations have identified PIP kinase activity on either the plasma membrane or in the cytosol. More recently, there have been several reports of PIP kinase activity in the nucleus (29-33). Whether the type I or II isoforms characterized thus far have distinct subcellular localizations has not been completely determined. Only the subcellular location of the type II kinase (9) has been studied. In addition to being found on the membrane and in the cytosol, the type II isoform is found in nuclei (30). Interestingly, only the β1 isoform of phospholipase C is found in nuclei (31-33), suggesting that the role of the phosphoinositides in nuclear signaling may be modulated through this enzyme. The varied localization of this isoform and the other phosphoinositide enzymes suggests the need for multiple mechanisms to modulate their activity or location. It also suggests a role for the involvement of the phosphoinositide kinases in many different cellular functions.

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REFERENCES
Type I PIP 5-Kinase Purification and Regulation