Phosphatidylinositol 4-phosphate 5-kinase type I is regulated through phosphorylation response by extracellular stimuli

Phosphatidylinositol 4-phosphate 5-kinase (PIPK) catalyzes a final step in the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$), a lipid signaling molecule. Strict regulation of PIPK activity is thought to be essential in intact cells. Here we show that type I enzymes of PIPK (PIPKI) are phosphorylated by cyclic AMP-dependent protein kinase (PKA), and phosphorylation of PIPK suppresses its activity. Serine 214 was found to be a major phosphorylation site of PIPK type Ia (PIPKIa) that is catalyzed by PKA. In contrast, lysophosphatidic acid-induced protein kinase C activation increased PIPKI activity. Activation of PIPKI was induced by dephosphorylation, which was catalyzed by an okadaic acid-sensitive phosphatase, protein phosphatase 1 (PP1).

In vitro dephosphorylation of PIPKIs with PP1 increased PIPK activity, indicating that PP1 plays a role in lysophosphatidic acid-induced dephosphorylation of PIPKI

These results strongly suggest that activity of PIPKIa in NIH 3T3 cells is regulated by the reversible balance between PKA-dependent phosphorylation and PP1-dependent dephosphorylation.

Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is a signal-generating phospholipid with crucial roles in various cellular processes. PIP$_2$ is the best substrate for phosphoinositide-specific phospholipase C, and PIP$_2$ hydrolysis generates two second messengers, 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate. Inositol 1,4,5-trisphosphate binds to specific receptors and induces release of calcium from intracellular stores (1), whereas DG activates protein kinase C (PKC) (2). In mammalian cells, PIP$_2$ can be further phosphorylated by phosphoinositide 3-kinase for generation of phosphatidylinositol 3,4,5-trisphosphate, a mediator of cell growth and survival (3, 4). In addition, PIP$_2$ directly modulates the activity of numerous enzymes and proteins involved in diverse cellular processes including exocytosis (5), cytoskeletal re-organization (6–8), and membrane trafficking (9).

Consistent with this important role played by PIP$_2$ in cellular signaling, intracellular PIP$_2$ levels are strictly regulated (10, 11). In response to various extracellular stimuli, a hydrolysis of PIP$_2$ has taken place, and its levels are rapidly decreased, resulting in the shortage of PIP$_2$. However, since compensatory synthesis of PIP$_2$ is rapidly induced, PIP$_2$ levels are constantly maintained, and the subsequent generation of second messengers inositol 1,4,5-trisphosphate and DG is renewed (12, 13).

PIP$_2$ is synthesized from phosphatidylinositol (PI) by two lipid kinases, phosphatidylinositol kinase (PIK) and PIPK. At least two immunologically distinct PIPK subtypes, PIPK type I and PIPK type II (13), exist in mammalian cells and are composed of three isoforms α, β, and γ (13–18). Most PIP$_2$ synthesis is catalyzed by type I enzymes, which phosphorylate the D-5 position of the inositol ring of PI 4-phosphate (PI4P). Recently, type II enzymes have been reported to be a PI 5-phosphate 4-kinase (19), which suggests the presence of an alternative pathway of PIP$_2$ synthesis catalyzing phosphorylation of PI 5-phosphate (PI5P).

A variety of stimuli, including GTPγS (20, 21), phorbol esters (22, 23), tyrosine phosphatase inhibitors (24), integrin (25), and EGF (26–28), have been reported to regulate PIP$_2$ synthesis. Type I enzymes were shown to be activated by acid phospholipids, especially phosphatidic acid (29), and to be regulated by GTP-binding proteins, Rho (30, 31) and Rac (32) in a manner dependent on GTPγS. In EGF-induced membrane ruffling, PIPKIs is physiologically downstream of the small G protein ADP-ribosylation factor, ARF6 (32). However, a lot of parts are not yet clear to explain how PIPK activity is regulated by extracellular stimulation during a few minutes in cells.

Considering this, we hypothesized that PIPKI itself is a substrate for some protein kinase in response to extracellular stimuli, which allows it to regulate the enzymatic activity. In particular, this is reasonable, since PIPKI from plasma membrane of Schizosaccharomyces pombe is phosphorylated by casein kinase I and is thereby inactivated (33), and activation of PKC by phorbol ester PMA treatment, which leads to phosphorylation of cellular proteins, stimulates PIP$_2$ synthesis (22, 23). Moreover, tyrosine phosphoproteins associated with EGF receptor have PI4K and PIPK activity (24, 28). Despite these data, there is still no direct evidence for regulation of PIPK activity by phosphorylation in mammalian cells. Thus, in this study, we examine whether there is a kinase that catalyzes PIPK phosphorylation and thereby regulates the activity.

Here we show that PIPKI is phosphorylated and inactivated by protein kinase A (PKA) both in vitro and in vivo, and we identify the PKA phosphorylation site of PIPKIa. Furthermore, we demonstrate that activation of PIPKIa in response to LPA treatment is induced by dephosphorylation of PIPKIa. Our
Phosphorylation of PIPKI by PKA

findings suggest that phosphorylation and dephosphorylation of PIPKIα are important for regulation of PIP₂ biosynthesis and phospholipid signaling in cells.

EXPERIMENTAL PROCEDURES

Materials—P4P was purified by neomycin column chromatography from crude phospholipids extracted from bovine spinal cord (34). [γ-32P]ATP and [35S]orthophosphate were from PerkinElmer Life Sciences. Polyvinylidene difluoride membranes for Western blot analysis were from Nihon Eido (Tokyo, Japan). Thin layer chromatography silica plates and cellulose plates for separation of phospholipids, phosphoamino acids, and phosphopeptides were from Merck. Monoclonal and polyclonal anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein kinase inhibitors H7, H89, KN-62, and genistein were purchased from Seikagaku Co. (Tokyo, Japan). Okadaic acid and calphostin C were from Wako Life Science Reagents (Tokyo, Japan). LPA and PMA were from Sigma. PKA catalytic subunit was from Promega (Madison, WI).

Cell Culture—NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum. Serum starvation was performed by culturing cells in Dulbecco’s modified Eagle’s medium without serum for 12–24 h.

Transient Expression and Stimulation of NIH 3T3 Cells—Full-length cDNAs encoding mouse PIPKIα, PIPKIβ, and PIPKIγ were ligated into the SalI-BamHI site of pCMV-Myc or the BamHI site of pEF-BOS-Myc mammalian expression vectors. NIH 3T3 cells were seeded into 60-mm dishes at a density of 2 × 10⁵ cells per dish and cultured overnight. 15 μg of expression plasmid was transfected into cells by the Ca²⁺ phosphate method (35). After incubation for 4 h, cells were washed with fresh medium and cultured for an additional 12 h. In stimulation experiments, the culture medium was aspirated and replaced with fresh serum-free medium for 12 h prior to initiation of metabolic ³²P labeling experiments. Various inhibitors were added throughout the preincubation for times indicated prior to addition of growth factors.

Measurement of PIPKI Activity—Expression plasmid-transfected NIH 3T3 cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 30 mM sodium pyrophosphate, 1% Nonidet P-40, 1 mM EDTA, 1% Triton X-100, 25 mM NaF, 0.1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride). The expressed enzyme was immunoprecipitated with monoclonal anti-Myc antibody and washed three times with lysis buffer and then once with reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EDTA). The reaction was started by adding 50 μM PIP₄, 50 μM ATP, and 1 μCi of [γ-32P]ATP in 50 μL. After incubation for 10 min at room temperature, the lipids were extracted with 1 N HCl for 10 min at 60 °C.

Activity—All assays were performed in the linear range with regard to the protein amounts and the incubation time in each assay system.

In Vitro Phosphorylation of PIPKI by PKA—Phosphorylation of PIPKI by PKA was carried out as described previously (36). 1 μg of GST-PIPKI protein was immobilized on 20 μl of glutathione-Sepharose beads and then incubated in 50 μl of 25 mM Tris-HCl pH 7.5, containing 5 mM MgCl₂, 2 mM EDTA, 1 μCi of [γ-32P]ATP, and 0.1 μg of PKA catalytic subunit for 30 min at room temperature. The reaction was stopped by addition of SDS sample buffer or ice-cold phosphate-buffered saline (PBS). The reaction mixture was centrifuged, and the beads were subsequently washed three times with PBS and then subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis.

Dephosphorylation of PIPKI by Alkaline Phosphatase—Myc-tagged PIPKI was immunoprecipitated from lysates of transfected NIH 3T3 cells. The immunoprecipitates were washed twice with lysis buffer and then once with alkaline phosphatase buffer (50 mM Tris-HCl, pH 8.2, 50 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Immunoprecipitates were subsequently resuspended in 2 μg of calf intestinal alkaline phosphatase (Takara Shuzo Co. Ltd., Tokyo, Japan) in 10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 50 mM KCl, 0.1 mM ZnCl₂, and 50% glycerol and incubated at 30 °C for 60 min.

Dephosphorylation of PIPKIα by Protein Phosphatase—1 μg of GST-PIPKIα immobilized on beads was phosphorylated with PKA and subsequently washed first with PBS and then dephosphorylation buffer (50 mM MOPS, pH 7.5, 1 mM MnCl₂, 150 mM NaCl, and 2 mM EDTA). The resulting beads were incubated in 50 μl of dephosphorylation buffer containing 0.5 units of PP1 or PP2A (Upstate Biotechnology Inc.) at 30 °C for 30 min.

Results

Metabolic ³²P Labeling and Phosphoamino Acid Analysis—NIH 3T3 cells were transfected with Myc-tagged PIPKIα and placed in fresh serum-free medium for at least 12 h prior to initiation of metabolic ³²P labeling experiments. Cells were subsequently incubated in phosphate-free Dulbecco’s modified Eagle’s medium for 2 h and then labeled in the same medium containing [³²P]orthophosphate (0.2 mM) for 6 h. After Bt₂cAMP treatment, cells were lysed in lysis buffer, and PIPKIα was immunoprecipitated with anti-Myc antibody. Immune complexes were washed once in lysis buffer and then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The radioactive band corresponding to PIPKIα was cut out of the gel and subjected to phosphoamino acid and tryptic peptide mapping. Phosphorylated proteins excised from gel bands were hydrolyzed in 6 N HCl for 3 h at 110 °C. The resulting amino acids, together with standard phosphoamino acids, were spotted onto cellulose thin layer plates and were separated by two-dimensional electrophoresis in 2.5% formic acid and 7.5% acetic acid, pH 1.9, and then in 5% acetic acid and 0.5% pyridine, pH 3.5. Labeled phosphoamino acids were detected by autoradiography. Positions of the standard phosphoamino acids were detected by ninhydrin staining.

Tryptic Peptide Mapping—Tryptic peptide mapping was performed as described previously (37). Labeled PIPKI was digested twice with 10 μg of tosylsulfonphenylalanyl chloromethyl ketone-treated trypsin. Released phosphopeptides were spotted onto cellulose thin layer plates and separated first by electrophoresis at 1,000 V in pH 1.9 buffer for 27 min and then by ascending chromatography in n-butyl alcohol/pyridine/acetic acid/water (75:50:15:60).

Results

PIPKI Is a Phosphoprotein—Anti-Myc antibody precipitates of Myc-tagged PIPKI expressed in NIH 3T3 cells appeared as broad bands on Western blots. Thus, we first examined whether these electrophoretic mobility shifts are caused by phosphorylation of PIPKI. After NIH 3T3 cells expressing Myc-PIPKIα, β, or γ were labeled with [³²P]orthophosphate, the cell lysates were immunoprecipitated with anti-Myc antibody and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 1, phosphorylation of all type I enzymes was evident in intact cells. These results show that the mobility shift of Myc-PIPKIα was caused by phosphorylation.

Phosphorylation of PIPKI by PKA in Vivo Suppresses PIPKI Activity—To identify the kinase chiefly responsible for phosphorylation of PIPKI in NIH 3T3 cells, the effects of various protein kinase inhibitors, the PKC inhibitor H7, the PKA inhibitor H89, the calmodulin dependent kinase inhibitor KN-62, and tyrosine kinase inhibitor genistein on PIPKIα phosphorylation.

I.B. α - Myc

autoradiography

Fig. 1. PIPKI is a phosphoprotein. Western blot and [³²P]orthophosphate labeling of PIPKI are shown. NIH 3T3 cells were expressed with Myc-tagged PIPKI three isoforms (Iα, Iβ, and Iγ) and then labeled with [³²P]orthophosphate (0.2 mM) for 6 h. Expressed PIPKI proteins were immunoprecipitated with anti-Myc antibody and subjected to SDS-polyacrylamide gel electrophoresis. The results were detected by Western blot analysis with anti-Myc antibody and autoradiography. I.B., immunoblot.
PIPKI is phosphorylated and suppressed by PKA in vivo. A, specific inhibition of PIPKIα phosphorylation by H89. NIH 3T3 cells were treated with Myc-PIPKIα and then treated with 50 μM H7, H89, KN62, or genistein (Gen.) for 30 min, respectively. Immunoprecipitated PIPKIs was subjected to Western blot analysis with anti-Myc antibody or PIPK activity assay. The kinase reaction was carried out in the presence of 50 μM P14P for 10 min at room temperature. cont., control. B, phosphorylation by PKA is conserved in all PIPKI isoforms. NIH 3T3 cells expressing Myc-PIPKI three isoforms were treated with 50 μM H89 for 30 min. Western blot analysis with anti-Myc antibody and PIPK activity assay were performed.

PKA Catalyzes Phosphorylation and Suppresses the Activity of PIPK in Vitro—The effect of PKA-catalyzed phosphorylation of PIPKIα, PIPKIβ, and PIPKIγ on their activities was tested in vitro with the corresponding GST-PIPKI fusion proteins expressed in and purified from Escherichia coli cells. Incubation of PIPKI proteins with the catalytic subunit of PKA and [γ-32P]ATP resulted in phosphorylation and 50% inhibition of all PIPKI subtypes (Fig. 3A).

In addition, treatment of Myc-PIPKIα immunoprecipitated from NIH 3T3 cell lysates with alkaline phosphatase clearly decreased the electrophoretic mobility of Myc-PIPKIα and increased its activity by about 170% (Fig. 3B). In contrast, incubation of the Myc-PIPKIα immunoprecipitates with PKA catalytic subunit did not significantly decrease PIPK activity, despite additional mobility shifts. The shift in mobility of PIPKIα by in vitro PKA phosphorylation was likely due to phosphorylation of physiologically irrelevant sites that are not phosphorylated in vivo and do not affect PIPK activity.

To determine directly the effect of phosphorylation on the activity of PIPK, activity of PIPKI was examined in a time-dependent manner during phosphorylation by PKA. After [γ-32P]ATP addition, the level of GST-PIPKIα phosphorylation was maximal in 20 min and sustained for at least 1 h. The inhibition time course of PIPKIα activity (maximally inhibited to 50% of the control) correlated closely with the phosphorylation time course by PKA, showing that activity of PIPKIα is regulated by PKA-dependent phosphorylation (Fig. 4).

PKA Catalyzes Phosphorylation of PIPKIα Ser-214—we next tried to characterize the phosphorylation sites of PIPKIα by PKA. Phosphoamino acid analysis of [32P]GST-PIPKIα, which was produced in vitro by incubation with PKA and [γ-32P]ATP, revealed predominant phosphorylation of only serine residues (Fig. 5). No phosphotyrosine was detected by Western blot analysis with PY-20 anti-phosphotyrosine antibody (data not shown). To identify further the PKA phosphorylation sites of PIPKIα, wild type and several C-terminal deletion mutants of PIPKIα were constructed and designated Iα full, Iα-(1–392), Iα-(1–260), and Iα-(1–175), respectively. The wild type and mutants were expressed in E. coli cells to produce GST fusion proteins. Purified GST proteins were incubated with the PKA catalytic subunit and [γ-32P]ATP, and fusion protein phosphorylation was quantified. As shown in Fig. 6A, deletion mutants Iα-(1–392) and Iα-(1–260) showed similar phosphorylation level to that of Iα full. In contrast, the Iα-(1–175) mutant, which lacks 365 C-terminal residues of PIPKIα, resulted in dramatically reduced phosphorylation, by only 3.5% of Iα full. These results suggest that the PKA phosphorylation sites exist within a central region, approximately 85 amino acids between 175 and 260 of PIPKIα. By interrogating the primary sequence of this region, we found that there are PKA phosphorylation site consensus sequence (RRXS) and serine residues conserved in all three PIPKI as shown in the results of Figs. 1, 2B, and 3A. Based on these results, serine-to-alanine point mutations were performed in PIPKIα that corresponded to putative PKA phosphorylation sites containing serine 207 or 214. Purified mutant and wild type PIPKIα proteins were incubated with PKA and [γ-32P]ATP, and the reaction products were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. No significant change in the level of phosphorylation was observed in the S207A mutant. In contrast, the phosphorylation level of S214A mutant was 60% less than that of wild type PIPKIα (Fig. 6B). Ser-214 was likely to be a major PKA phosphorylation site in PIPKIα.

To confirm this, peptide-map analysis was performed on tryptic cleavage fragments of wild type and mutant proteins phosphorylated by PKA in vitro. Three major phosphopeptides were detected in two-dimensional maps of wild type PIPKIα tryptic fragments (spots 1–3 in Fig. 6C). However, in maps of S214A mutant, spots 2 and 3 had disappeared. These two spots appear to be derived from incomplete digestion products by trypsin, whereas spot 1 was not changed. However, surpis-
ingly, tryptic peptide maps of PIPKÎ± expressed in NIH 3T3 cells resulted in only two phosphopeptides, which have disappeared in maps of S214A mutant. In addition, treatment of the cells with Bt2cAMP neither induced phosphorylation of additional sites nor significantly increased the phosphorylation level of Ser-214 (data not shown). Therefore, we conclude that PIPKÎ± exists as the Ser-214 phosphorylated form in most NIH 3T3 cells. Western blot analysis using lysates of NIH 3T3 cells expressing S214A mutant showed that this mutated PIPKÎ± protein had migrated to the lowest mobile band (data not shown).

**Mechanism of LPA-induced Activation of PIPKÎ±**—It is well known that PIPK activity is increased in response to various extracellular stimuli. However, little is known about the signal pathway that leads to PIPKÎ± activation. Thus, we first examined how phosphorylation of PIPKÎ± is changed in response to extracellular stimuli. As shown in Fig. 7A, stimulation of NIH 3T3 cells with LPA induced a transient but clear suppression of PIPKÎ± phosphorylation, which peaked at 3 min and then rapidly declined. Good correlation was observed between PIPKI dephosphorylation and activation. Since LPA activates pathways involving PKC and/or Ca\(^{2+}\) signaling in a variety of cells (43–46), NIH 3T3 cells were treated with the phorbol ester PMA, an activator of PKC. This treatment induced a strong dephosphorylation, and PIPKÎ± activity increased by about 150% of the untreated control at 30 min (Fig. 7B). However, as shown in Fig. 7C, treatment of NIH 3T3 cells with calphostin C, a selective PKC inhibitor, completely blocked PIPKÎ± dephosphorylation in response to LPA.

To assess the requirement of Ca\(^{2+}\) in this regulation, we next used a combination of EGTA and 1,2-bis(2-aminophenoxy)ethane-N\(_2\)N\(_2\)N\(_2\)N\(_2\)-tetraacetic acid-AM, antagonist of Ca\(^{2+}\) mobilization and Ca\(^{2+}\) entry. Interestingly, LPA-induced dephosphorylation and activation of PIPKÎ± was not different (data not shown). Ionophore, A23187, treatment also had no effect (data not shown). Taken together, these results suggest that a
PKC-dependent pathway, not a Ca\(^{2+}\)-dependent pathway, played a key role in the dephosphorylation and activation of PIPKI\(\alpha\) in response to LPA.

Furthermore, we examined whether major serine/threonine phosphatases such as protein phosphatase type 1 (PP1) and protein phosphatase type 2 (PP2A) are involved in the LPA-induced dephosphorylation of PIPKI\(\alpha\). Pretreatment of NIH 3T3 cells with okadaic acid (OA), a potent inhibitor of PP1 and PP2A, also completely blocked dephosphorylation and activation by LPA (Fig. 7C), suggesting that an OA-sensitive phosphatase, PP1 or PP2A, presumably regulates dephosphorylation and activation of PIPKI\(\alpha\) in response to LPA stimulation.

**PP1 Dephosphorylates and Activates PIPKI\(\alpha\)**—To examine whether OA-sensitive phosphatase directly participates in dephosphorylation and activation of PIPKI\(\alpha\), dephosphorylation assays were performed with \[^{32}\text{P}]\text{ATP} in vitro. As shown in Fig. 8, PP1 clearly dephosphorylated and activated PIPKI\(\alpha\). \[^{32}\text{P}]\text{Labeled GST-PIPKI}\(\alpha\) treated with PP2A was also found to be dephosphorylated; however, its activity remained unchanged (data not shown). Taking these results together, we conclude that activation via dephosphorylation of PIPKI\(\alpha\) is catalyzed by PP1.

**DISCUSSION**

In the present study, we show that PIPKI is a phosphoprotein, and the PIPKI phosphorylation state is dynamically regulated by the opposing actions of a PIPKI kinase and a PIPKI phosphatase in response to external stimuli (Fig. 9). Most of the PIPKI\(\alpha\) expressed in NIH 3T3 cells existed in fully phosphorylated form by PKA at serine 214 even in the resting cells, which was likely to allow PIPKI\(\alpha\) to be in an inactive state. In fact, when NIH 3T3 cells expressing PIPKI\(\alpha\) were stimulated with Bt2cAMP, we saw no significant mobility shift (data not shown). However, potential stimuli such as LPA led to dephosphorylation and subsequent activation of PIPKI\(\alpha\). PP1 was...
identified as a primary candidate for LPA-mediated PKC-dependent phosphatase. In addition, from the results of Fig. 3B and in vitro dephosphorylation with PP2A, PP2A is likely to function at another phosphorylation site, not Ser-214, that is phosphorylated by PKA only in vitro.

Ser-214 is a major phosphorylation site of PIPKια by PKA in vitro and in vivo. This site is conserved in both type I (14) and type II isoenzymes (18). However, type II enzymes do not have perfect PKA phosphorylation site consensus sequences, and we did not detect any mobility shift of type IIα by Western blot analysis after treatment with either H89 or Bt2cAMP. This is consistent with results of earlier studies showing that PIPKιγ is phosphorylated by a serine kinase but not by PKA or PKC (18). Thus, in NIH 3T3 cells, activity regulation via phosphorylation of PIPK type I is more likely to be important for maintaining intracellular total PIP2 levels because PI4P, the major substrate for PIPKι, is extremely abundant than PI5P, the substrate for PIPK type II (19).

Several groups reported that PIP2 synthesis is regulated by extracellular stimuli. Treatment of HEK-293 cells with tyrosine kinase inhibitor alters PIP2 levels (38), and Rho protein and phospholipase D activation appear to be involved in this process. However, involvement of PIPK was also remained unclear, and in the present study, we did not detect any change in phosphorylation or activation of PIPKια in NIH 3T3 cells in response to treatment with the tyrosine kinase inhibitor, genistein. Tyrosine-phosphorylated proteins immunoprecipitated with anti-EGF antibody from EGF receptor-transfected mouse cells or A431 cells, an epidermoid carcinoma cell line overexpressing EGF receptors, showed PIK activity and PIPK activity (24, 28). However, in any case, tyrosine phosphorylation by itself did not alter the activity of PIPK. Thus, it seems likely that some other tyrosine kinase or phosphotyrosyl protein interacts with both EGF receptor and PIK or PIPK.

Although involvement of PKC in the inositol phospholipid metabolism has been also investigated in many reports, the exact role PKC plays is not well understood. PMA treatment increased PIP2 levels 1.5–2.5-fold in lymphocytes (23) and in human platelets (22) but did not stimulate PIP2 hydrolysis by phospholipase C. Thus, direct effect on inositol lipid kinase or phosphatase through activation of PKC was suggested (22). Our results in this study provided evidence suggesting that PKC, which is usually activated by the PIP2 hydrolysis product DG, contributes to maintenance of PIP2 level through a feedback regulation of PIPKI in response to receptor activation.

IP2 synthesis is also regulated by GTP-binding proteins. Phosphatidic acid (PA)-stimulated PIPK activity was found to be associated with Rac in a GTPαS-dependent manner in liver and Swiss 3T3 cells (39), and interaction between Rho and PIPK activity was demonstrated in mouse fibroblast cell lines (30, 31). In addition, the ADP-ribosylation factor 1 (ARF1) interacts with PIPK. Godi et al. (40) and Jones et al. (41) recently showed that PIPK and PI4Kβ are direct effectors of ARF1. ARF1 activated by GTPγS recruits PIPKI and PI4Kβ to the Golgi complex, resulting in a potent stimulation of PIP2 synthesis in Golgi membrane, and these effects were independent of the known activities of ARF on a coat proteins and phospholipase D. At the Golgi membranes, PIP2 stimulates not only guanine nucleotide exchange factor for ARF1 but also a GTPase-activating factor for ARF1 in a PA-dependent manner. Thus, PIP2 up-regulates and down-regulates ARF activity and, consequently, vesicular trafficking in Golgi membrane. Therefore, control of PIP2 levels at this cellular compartment is essential. Phosphorylation of PIPKI by PKA may reduce PIP2 resynthesis in Golgi membrane and participate in feedback regulation of ARF1 activity. In fact, when we tested the effect of PA on the activity of PIPK, inhibition of PIPK activity by phosphorylation was not affected by PA (data not shown).

Recently, Martin et al. (42) reported that activation of PKA induces binding of ARF1 to Golgi membranes, and this binding is mediated by an unknown target protein, which is phosphorylated by PKA and dephosphorylated by OA-sensitive phosphatase. In addition, this step is not dependent on guanine nucleotides. Therefore, we speculate that the target protein that is phosphorylated by PKA is PIPKI and that PIP2 formed by PIPKI promotes stable association of ARF1 with the Golgi membrane. Instead of guanine nucleotide factors, PKA-depend-
ent phosphorylation of PIPKI is likely to regulate activity of ARF1 and thereby control its association with Golgi membrane. That is, PIPKI first increases recruitment of ARF1 in Golgi membrane through PIP2 generation, and then inactivation of PIPKI by PKA-dependent phosphorylation would likely promote the generation or extraction of trafficking vesicles, thereby subsequently stimulating the ARF recruitment cycle to Golgi membrane. We expect that further studies with antibodies that specifically recognize PIPKI phosphorylated at Ser-214 will help address this speculation.

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Phosphorylation of PIPKI by PKA

4787