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Phosphatidylinositol-4-phosphate 5-Kinase Isoforms Exhibit Acyl Chain Selectivity for Both Substrate and Lipid Activator*

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Background: Do isoforms of phosphatidylinositol-4-phosphate 5-kinase favor specific lipids?
Results: The enzymes favor substrates and activators with specific acyl chains, which are different for substrates and activators.
Conclusion: The γ isoform is the most selective for different acyl chains.
Significance: Selectivity of phosphatidylinositol-4-phosphate 5-kinases for acyl chains could be part of a tightly regulated mechanism producing physiologically active PtdIns(4,5)P_2 species in the cell.

Phosphatidylinositol 4,5-bisphosphate is mostly produced in the cell by phosphatidylinositol-4-phosphate 5-kinases (PIP5K) and has a crucial role in numerous signaling events. Here we demonstrate that *in vitro* all three isoforms of PIP5K, α, β, and γ, discriminate among substrates with different acyl chains for both the substrates phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol (PtdIns) although to different extents, with isoform γ being the most selective. Fully saturated dipalmitoyl-PtdIns4P was a poor substrate for all three isoforms, but both the 1-stearoyl-2-arachidonoyl and the 1-stearoyl-2-oleoyl forms of PtdIns4P were good substrates. V_{max} was greater for the 1-stearoyl-2-arachidonoyl form compared with the 1-stearoyl-2-oleoyl form, although for PIP5Kβ the difference was small. For the α and γ isoforms, K_m was much lower for 1-stearoyl-2-oleoyl PtdIns4P, making this lipid the better substrate of the two under most conditions. Activation of PIP5K by phosphatidic acid is also acyl chain-dependent. Species of phosphatidic acid with two unsaturated acyl chains are much better activators of PIP5K than those containing one saturated and one unsaturated acyl chain. PtdIns is a poor substrate for PIP5K, but it also shows acyl chain selectivity. Curiously, there is no acyl chain discrimination among species of phosphatidic acid in the activation of the phosphorylation of PtdIns. Together, our findings indicate that PIP5K isoforms α, β, and γ act selectively on substrates and activators with different acyl chains. This could be a tightly regulated mechanism of producing physiologically active unsaturated phosphatidylinositol 4,5-bisphosphate species in the cell.

The phosphatidylinositol phosphate kinases have a multitude of important roles in cell signaling (1–3). This family of enzymes is responsible for the regulation of cytoskeleton dynamics, vesicular trafficking, and cell migration as well as transcription control at the nucleus. The headgroup specificity of these enzymes has been extensively investigated with regard to the number and position of phosphate groups required on the substrate, as well as the position on the inositol that is phosphorylated by each of these enzymes. However, there has been very little investigation regarding the role of the acyl chains in the substrate specificity of these enzymes. In some studies, natural forms of the substrates were used, whereas in other studies, dipalmitoylated lipids were used because of their greater stability and commercial availability. Except for the presence of dipalmitoyl phosphatidylcholine in certain organs, dipalmitoyl lipids are present in very low abundance in biological tissues. We recently showed that the dipalmitoylated form of phosphatidylinositol 4-phosphate (PtdIns4P)[2] was a much poorer substrate for phosphatidylinositol-4-phosphate 5-kinase (PIP5K) than the natural form of PtdIns4P (4), demonstrating some acyl chain specificity in the action of this enzyme on substrates.

In the current study, we focused on isoforms of PIP5K that catalyze the phosphorylation of PtdIns4P to form the important secondary messenger phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (5). There are three isoforms of PIP5K given the designations α, β, and γ. Each PIP5K isoform produces multiple splicing variants (6–9). Although all three isoforms have a high degree of homology and all catalyze the same reaction, each appears to have some unique properties. PIP5Kα promotes the depolymerization of neuronal microtubules (10). The α isoform suppresses phagocytosis and accumulates transiently on forming phagosomes (11). This isoform also appears in PDGF-induced membrane ruffles in platelets (12). PIP5Kα also interacts directly with diacylglycerol kinase ζ (DGKζ), resulting in the promotion of the formation of PtdIns(4,5)P_2, probably through the activation of PIP5K by phosphatidic acid (PA), the

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[2] The abbreviations used are: PtdIns4P, phosphatidylinositol-4-phosphate; DGK, diacylglycerol kinase; PtdIns, phosphatidylinositol; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; PA, phosphatidic acid. For the abbreviations of the variety of lipids with specific acyl chains used in this work, see Table 1.

product of the reaction catalyzed by DGKζ (13, 14). The β isoform of PIP5K is activated by both Ser/Thr and by Tyr phosphorylation that is promoted by oxidative stress (15). This isoform controls neutrophil polarity and directional movement (16, 17). The γ isoform of PIP5K affects cell to cell contacts, and its activity correlates with a poor prognosis for breast cancer (18, 19). This isoform also regulates distinct stages of Ca^{2+} signaling in mast cells (20). PIP5Kγ is also the predominant isoform for producing PtdIns(4,5)P_2 in the brain (21, 22).

Enzymatic activity of all three PIP5Ks was shown to be strongly activated by PA (23), produced either through phospholipase D (PLD) or several isoforms of DGK (8, 24). There has been only limited assessment of the role of the acyl chains of PA in this activation. Activation by PA of the enzyme that synthesizes PtdIns(4,5)P_2 as part of the PtdIns cycle, PIP5K, is particularly interesting because both PA and PtdIns(4,5)P_2 are lipid intermediates in the PtdIns cycle, and as intermediates in this cycle, they are highly enriched in stearoyl and arachidonoyl acyl chains. There is thus potential for a forward feedback activation of the PtdIns cycle by PA activating PIP5K.

EXPERIMENTAL PROCEDURES

Materials—SO-PtdIns4P and SA-, SO-, SL- and DL-PtdIns were custom-synthesized by Avanti Polar Lipids. As a source of SA-PtdIns4P, brain PtdIns4P (Avanti Polar Lipids) was used. DP-PtdIns4P was purchased from Echelon Biosciences Inc. All PAs were purchased from Avanti Polar Lipids. The abbreviations, full names, and alternative notations of all lipids used in this study are listed in Table 1.

PIP5K Constructs—HA-PIP5K isoform α and γ expression vectors were prepared as described previously (9, 25). HA-PIP5K isoform β expression vector was a kind gift of Drs. Santos Mañes and Rosa Ana Lacalle (Centro Nacional de Biotecnología, Madrid, Spain). c-Myc-PIP5Kα expression vector was prepared as described previously (13). HA-PIP5Kα and c-Myc-PIP5Kα correspond to the human form of the respective enzyme, splicing variant 2; HA-PIP5Kβ corresponds to the mouse form (96% protein homology with human PIP5Kβ); HA-PIP5Kγ corresponds to the human form, splicing variant 1 (640 amino acids). The mutants of c-Myc-PIP5Kα were designed using the QuikChange Lightning Kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. FLAG-PIP5Kα D322A expression vector (which corresponds to the human form of the enzyme) was prepared and tested as described previously (25–27). The presence of the desired mutations was verified by sequencing analysis.

Cell Culture—COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (Invitrogen) at 37 °C in an atmosphere of 5% CO_2. The cells were grown to about 80% confluence and transiently transfected with the expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested 48 h after transfection by scraping them into 1× PBS containing 1:100 protease inhibitor mixture for use with mammalian cells and tissue (Sigma-Aldrich). The cells were pelleted at 5000 × *g* at 4 °C and kept at −90 °C until further use.

Enzyme Preparations for Enzymatic Activity Assay—Cell pellets of COS-7 cells overexpressing one of the PIP5K proteins were resuspended in ice-cold cell lysis buffer (2% (v/v) (octylphenoxy)polyethoxyethanol (Nonidet P-40), 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin and leupeptin, 1 mM PMSF, 5 mM NaF, 100 μg/ml soybean trypsin inhibitor, and 1:100 protease inhibitor mixture for use with mammalian cells and tissue (Sigma-Aldrich)), allowed to lyse for 10 min on ice, sonicated for 10 min, and then incubated with agarose beads conjugated with anti-HA (sc-7392 AC, Santa Cruz Biotechnology, Inc.) or anti-c-Myc antibodies (sc-40 AC, Santa Cruz Biotechnology, Inc.) at 4 °C overnight. After that, the beads were centrifuged and washed one time with IP kinase buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Triton X-100); one time with PBS, pH 6.0, 0.5% Triton X-100; 1 time with 25 mM Tris, pH 8, 100 mM NaCl, 0.1% Triton X-100; one time with 25 mM Tris, pH 7.5, 500 mM NaCl, 0.1% Triton X-100; and one time with IP kinase buffer (28). After the final wash, the beads were briefly centrifuged and resuspended in 1× assay buffer. Purity of the PIP5K immunoprecipitate was confirmed by Coomassie Blue staining of the gel.

For preparation of a sample containing PIP5Kα heterodimer, cell pellets of COS-7 cells co-transfected with HA-PIP5Kα and FLAG-PIP5Kα D322A vectors were resuspended in ice-cold cell lysis buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 10 μg/ml aprotinin and leupeptin, 1 mM PMSF, 5 mM NaF, 100 μg/ml soybean trypsin inhibitor, and 1:100 protease inhibitor mixture for use with mammalian cells and tissue (Sigma-Aldrich)), allowed to lyse for 20 min on ice, and centrifuged at 12,000 × *g* for 10 min at 4 °C. The lysate was precleared with mouse IgG-agarose (Sigma-Aldrich) and then incubated with agarose beads conjugated with OctA probe (sc-807 AC; Santa Cruz Biotechnology, Inc.) for 5 h at 4 °C. After that, the beads were centrifuged and washed five times with TBS buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$). After the final wash, the beads were briefly centrifuged and resuspended in 1× assay buffer. The presence of both HA-PIP5Kα and FLAG-PIP5Kα D322A proteins in the immunoprecipitate was confirmed by Western blotting.

Immunoblot Analysis—Amounts of protein in the immunoprecipitates from transfected COS-7 cells were determined by immunoblotting as described previously (4). The membranes were incubated with either a 0.5 μg/ml concentration of mouse THE™ anti-HA tag IgG1 (GenScript, A01244) or a 1:800 dilution of mouse anti-c-Myc (sc-40; Santa Cruz Biotechnology, Inc.) as the primary antibody and a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse (sc-2005; Santa Cruz Biotechnology, Inc.) as the secondary antibody.

Quantification of Phospholipids PA, PtdIns4P, and PtdIns—The concentrations of all PA, PtdIns4P, and PtdIns stocks used in this study were determined experimentally based on an assay for inorganic phosphate as described previously (4, 29).

Detergent-Phospholipid-Mixed Micelle-based PIP5K Enzymatic Activity Assay—PIP5 kinase activity assay was performed as described by Parker *et al.* (30) with the following modifications. Mixed micelles were formed by hydrating the lipid films, composed of the substrate (PtdIns4P or PtdIns) with or without

TABLE 1
Lipids used and/or referred to in this study

Abbreviation	Full name	Alternative notation (*sn*-1/*sn*-2)
PA		
AAPA	1-Arachidoyl-2-arachidonoyl phosphatidic acid	20:0/20:4 PA
DAPA	1,2-Diarachidonoyl phosphatidic acid	20:4/20:4 PA
DLPA	1,2-Dilinoleoyl phosphatidic acid	18:2/18:2 PA
DOPA	1,2-Dioleoyl phosphatidic acid	18:1/18:1 PA
SAPA	1-Stearoyl-2-arachidonoyl phosphatidic acid	18:0/20:4 PA
SOPA	1-Stearoyl-2-oleoyl phosphatidic acid	18:0/18:1 PA
PtdIns		
DL-PtdIns	1,2-Dilinoleoyl phosphatidylinositol	18:2/18:2 PtdIns
SA-PtdIns	1-Stearoyl-2-arachidonoyl phosphatidylinositol	18:0/20:4 PtdIns
SL-PtdIns	1-Stearoyl-2- linoleoyl phosphatidylinositol	18:0/18:2 PtdIns
SO-PtdIns	1-Stearoyl-2-oleoyl phosphatidylinositol	18:0/18:1 PtdIns
PtdIns4P		
DP-PtdIns4P	1,2-Dipalmitoyl phosphatidylinositol-4-phosphate	16:0/16:0 PtdIns4P
SA-PtdIns4P	1-Stearoyl-2-arachidonoyl phosphatidylinositol-4-phosphate	18:0/20:4 PtdIns4P
SO-PtdIns4P	1-Stearoyl-2-oleoyl phosphatidylinositol-4-phosphate	18:0/18:1 PtdIns4P

the addition of PA (see Table 1 for the list of lipids used and their abbreviations), with 2× assay buffer and subsequently vortexing the hydrated lipid film for 2 min. Reactions were performed in a 100-μl reaction volume in an assay buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 0.1% Triton X-100, and 50 μM [γ-^{32}P]ATP (2 μCi/reaction). The reaction was stopped after 10 min by the addition of 500 μl of 1 N HCl and 2 ml of chloroform/methanol (1:1) simultaneously. The assay was washed twice with 1 ml of methanol, 1 N HCl (1:1). An aliquot of the organic layer was used to quantify the incorporation of ^{32}P into the lipid product using Cerenkov counting. Negative controls were run with the addition of beads immunoprecipitated from mock-transfected COS-7 cells and were confirmed to have activity levels significantly below immunoprecipitates from cells overexpressing PIP5K. Results are presented as the mean ± S.D. It was shown previously that substrate binding by PIP5Ks follows the surface dilution kinetic model described by Hendrickson and Dennis (70). Therefore, in this study, the substrate and PA concentrations are presented as the effective concentration of the substrate or PA at the surface of the micelle. The effective surface concentration of the substrate (C_{eff}) was calculated by multiplying the molar fraction of the substrate at the surface of the micelle by the total concentration of the substrate (28).

Kinetic Analysis of the Micelle-based Assay of PIP5K Activity—Kinetic parameters were calculated using the effective concentration of the substrate at the surface of the micelle following the formula from Jarquin-Pardo *et al.* (28). Using this treatment, the data fit Michaelis-Menten kinetics. The Michaelis-Menten constants, $V_{\max}$ and K_m, were evaluated by a nonlinear regression analysis (initial velocity (v_0) *versus* substrate concentration ([S])) using the GraphPad Prism software program (version 5.00).

RESULTS

PIP5Ks Are Sensitive to the Acyl Chain Composition of Substrate PtdIns4P—To determine if PIP5K isoforms discriminate between PtdIns4P with different acyl chain compositions, we compared the activity of PIP5K isoforms α, β, and γ with three different substrates, SA-PtdIns4P, SO-PtdIns4P, and DP-PtdIns4P (see Table 1 for lipid abbreviations). Our results showed that all isoforms exhibit a significant preference for the two substrates containing an unsaturated acyl chain (SA- and SO-PtdIns4P) compared with the substrate with only saturated acyl chains (DP-PtdIns4P) (Fig. 1, *A–C*). At low substrate concentrations (C_{eff} = 0.23 μM), PIP5Ks have preference for SO-PtdIns4P over SA-PtdIns4P, with the PIP5Kγ isoform showing the largest difference between these two substrates (Fig. 1, *A–C*). However, at higher substrate concentrations ($C_{\text{eff}} > 2$ μM for PIP5Kα and -β, $C_{\text{eff}} > 4$ μM for PIP5Kγ), the enzyme activity is higher for SA-PtdIns4P than for SO-PtdIns4P (Fig. 1, *D–F*).

If certain isoforms of PIP5K preferentially phosphorylated SA-PtdIns4P, it would suggest that this isoform is involved in the PtdIns cycle, contributing to the enrichment of phosphatidylinositols with the 1-stearoyl-2-arachidonoyl species. Kinetic analysis determined that PIP5K isoforms α and γ have a significantly lower K_m for SO-PtdIns4P than for SA-PtdIns4P, whereas PIP5Kβ has a similar K_m for both substrates (Table 2). The $V_{\max}$ parameter is higher for SA-PtdIns4P for all isoforms of PIP5K, although PIP5Kβ shows only a marginal difference (Table 2). As a result, the $V_{\max}/K_m$ value is the same, within error, for the three isoforms. The $V_{\max}/K_m$ parameter also corresponds to the rate constant at low substrate concentration.

Together these findings indicate that all isoforms of PIP5Ks (with isoform β to a smaller extent) distinguish among different acyl chains of PtdIns4P. The acyl chain selectivity of the PIP5Ks is large when there is a large difference in acyl chain structure, such as DP- *versus* SA- or SO-PtdIns4P species.

PIP5K Activation by PA Depends on the Acyl Chain Composition of both Substrate and Activator—Previously, we showed that PIP5K isoform α is sensitive to the acyl chain composition of phosphatidic acid and that the extent of PA activation is different for SA-PtdIns4P and DP-PtdIns4P (4). To determine if all isoforms of PIP5K exhibit similar acyl chain preference for PA, we compared the activation of PIP5K isoforms α, β, and γ by different species of PA (Fig. 2). Because acyl chain length and saturation of SA-PtdIns4P and DP-PtdIns4P differ significantly, we also tested SO-PtdIns4P as a substrate because it has

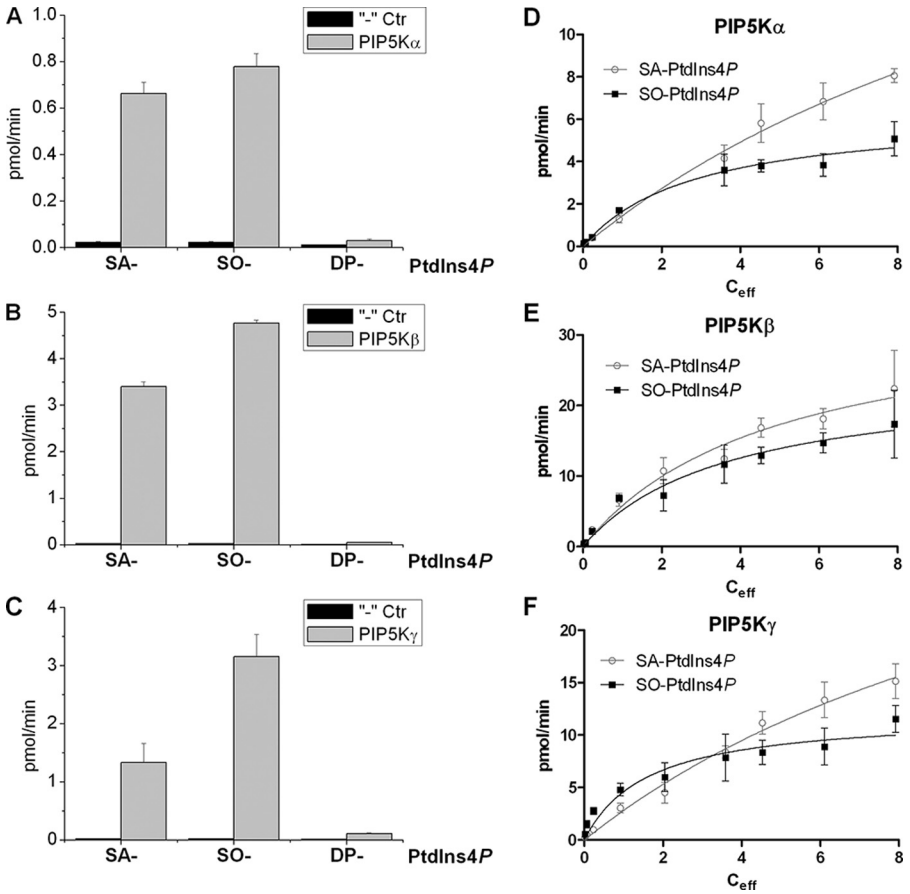

FIGURE 1. **HA-PIP5K isoforms α, β, and γ show sensitivity to the acyl chain composition of PtdIns4P substrate.** *A–C*, comparison of PIP5K activities with SA-, SO-, and DP-PtdIns4P at low substrate concentrations (total substrate concentration = 20 μM, equal to C_{eff} = 0.23 μM). The effective surface concentration (C_{eff}) of the substrate was calculated by multiplying the molar fraction of the substrate at the surface of the micelle by the total concentration of the substrate (28). *D–F*, comparison of PIP5K activities with SA-, and SO-PtdIns4P over a wide range of substrate concentrations (C_{eff} from 0.015 to 7.91 μM). *Error bars*, S.D.

TABLE 2

Summary of the kinetic parameters for HA-PIP5K isoforms α, β, and γ

Kinetic parameters are calculated using the effective concentration of PtdIns4P at the surface of the micelle. The effective surface concentration of PtdIns4P was determined by multiplying the molar fraction of PtdIns4P at the surface of the micelle by the total concentration of PtdIns4P. Values of V_{max} are relative values because the absolute amount of enzyme in the cell preparations is not known. Results are presented as the mean ± S.D.

Isoform	Substrate	K_m	V_{max}	V_{max}/K_m
		μM	*pmol min*$^{-1}$	μM^{-1} *min*$^{-1}$
HA-PIP5Kα	SA-PtdIns4P	16 ± 5	25 ± 5	1.5 ± 0.6
	SO-PtdIns4P	2.8 ± 0.9	6.3 ± 0.7	2.2 ± 0.7
HA-PIP5Kβ	SA-PtdIns4P	4.9 ± 1.4	34 ± 5	6.9 ± 2.2
	SO-PtdIns4P	3.7 ± 1.1	24 ± 3	6.6 ± 2.2
HA-PIP5Kγ	SA-PtdIns4P	15 ± 4	44 ± 10	3.0 ± 1.2
	SO-PtdIns4P	1.6 ± 0.6	12 ± 1	7.5 ± 3.1

the same *sn*-1 acyl chain as SA-PtdIns4P (18:0) but a different *sn*-2 acyl chain.

Our results showed that all three isoforms of PIP5K have similar profiles of PA activation but differ in the extent of activation, with isoform α being activated the most and isoform β activated the least with all three tested substrates (Fig. 2). When DP-PtdIns4P is used as a substrate, diarachidonoyl phosphatidic acid (DAPA) is undoubtedly the best activator of all PIP5Ks (Fig. 2, *G–I*). Further, the extent of DAPA activation is much higher than when other substrates are used (28-, 12-, and 24-fold for PIP5K isoforms α, β, and γ, respectively, when DP-PtdIns4P is used as a substrate). When SA-PtdIns4P is used as a substrate, dilinoleoyl-PA (DLPA) has a tendency to be a better activator, especially for PIP5Kα (Fig. 2, *A–C*). For SO-PtdIns4P, the profile of PA activation is somewhat similar to that of SA-PtdIns4P, but there is no significant preference for DLPA over other PAs with two unsaturated acyl chains (Fig. 2, *D–F*). Surprisingly, the only tested species of PA that does not activate all PIP5Ks is SOPA, and in most cases, SAPA is the next least potent activator (Fig. 2).

Thus, PIP5K isoforms α, β, and γ differ in the degree of PA activation, but all of them clearly discriminate between the acyl chains of both the substrate and the activator. The presence of a saturated acyl chain at the *sn*-1 position of PA considerably lowers the extent of activation. PIP5Ks have been implicated in a variety of distinct cellular processes, such as polarized trafficking of integrins (31) and regulation of polyadenylation of mRNAs (26, 32). It also has been suggested that different PIP5K isoforms may regulate endocytosis of different types of cargo (33). Therefore, variations in the acyl chain sensitivity and degree of PA activation could be a way of commitment of different isoforms to distinct cellular pathways.

PIP5Ks Are Sensitive to the Acyl Chain Composition of Substrate PtdIns—We next examined whether PIP5Ks are sensitive to the acyl chain composition of other substrates, such as PtdIns. First, we compared the activity of PIP5K with PtdIns4P and PtdIns as substrates. Our data confirm that *in*

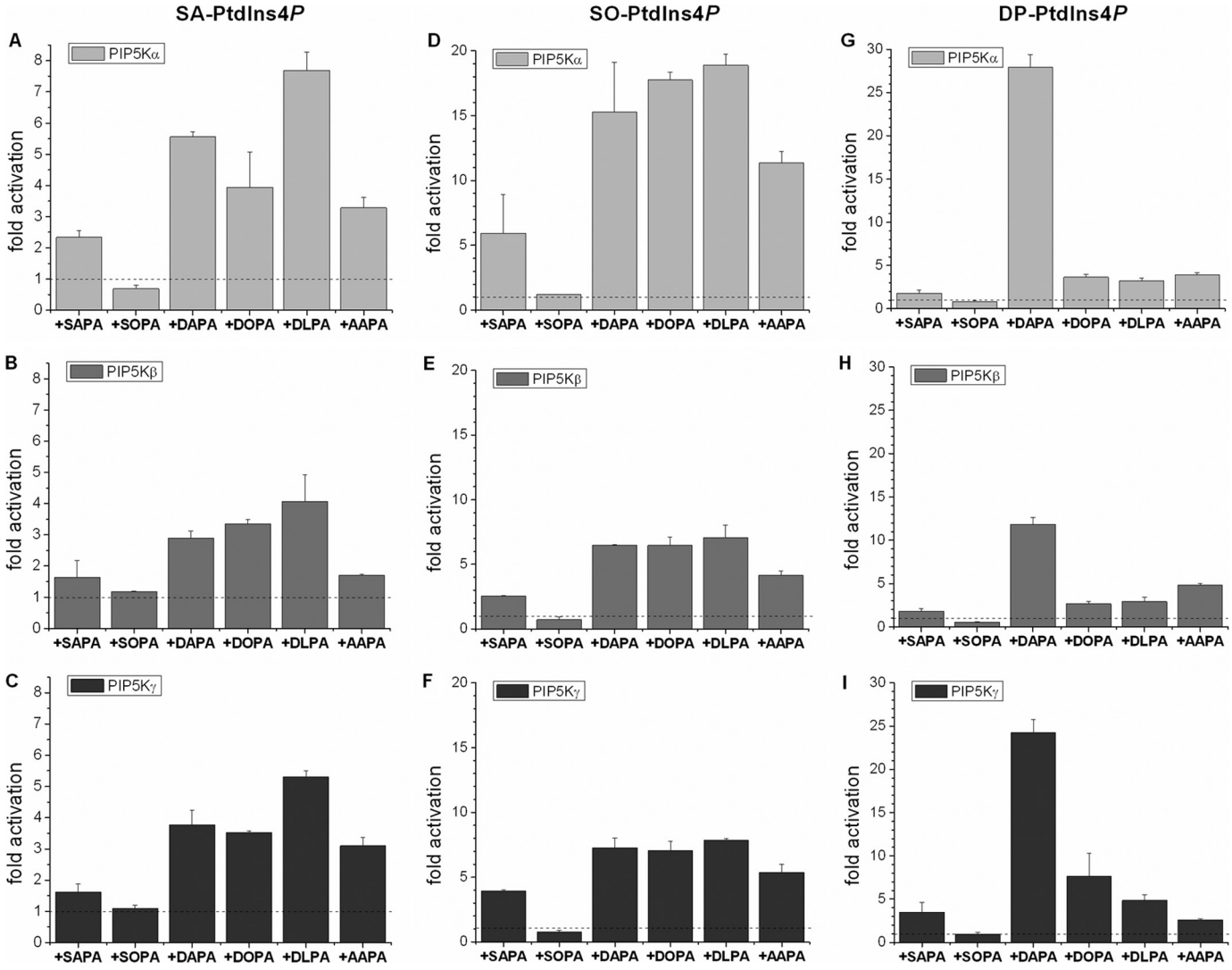

FIGURE 2. **Activation of HA-PIP5K isoforms α, β, and γ by different PAs with SA-PtdIns4P (*A–C*), SO-PtdIns4P (*D–F*), and DP-PtdIns4P (*G–I*) as substrates.** PIP5K enzymatic activity was measured with 10 μM (equal to C_{eff} = 0.06 μM) PtdIns4P and 50 μM (equal to C_{eff} = 1.42 μM) PA. *Error bars*, S.D.

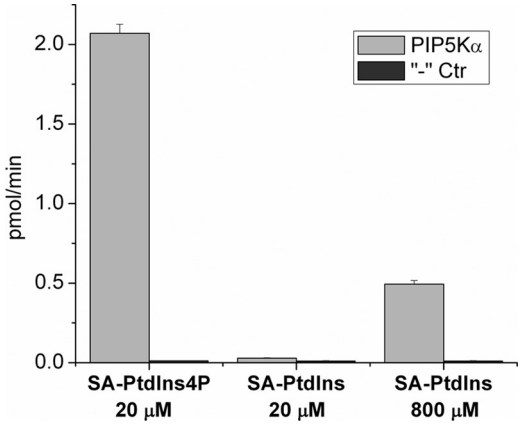

FIGURE 3. **PIP5Kα has a strong preference for PtdIns4P as a substrate over PtdIns.** PIP5K enzymatic activity was measured with either 20 μM SA-PtdIns4P, 20 μM SA-PtdIns (equal to C_{eff} = 0.23 μM), or 800 μM (equal to C_{eff} = 256 μM) SA-PtdIns. *Error bars*, S.D.

vitro PIP5Ks phosphorylate PtdIns4P at a much higher rate than PtdIns (Fig. 3) (34). For PIP5Kα with SA-PtdIns as a substrate, we determined the K_m parameter to be significantly higher (5 times) than for SA-PtdIns4P (K_m(SA-PtdIns) = 127 ± 36 μM) and determined V_{max} to be much lower (V_{max}(SA-PtdIns) = 0.14 ± 0.01 pmol/min *versus* V_{max}(SA-PtdIns4P) = 25 ± 5 pmol/min).

To test the acyl chain preference of PIP5Ks for PtdIns, we compared their enzyme activities with four different PtdIns species, SA-, SO-, SL-, and DL-PtdIns (see Table 1 for lipid abbreviations). The results show that all isoforms of PIP5Ks exhibit preference for SO- and SL-PtdIns, with isoform γ showing the strongest discrimination toward SO-PtdIns (Fig. 4). These data are in good agreement with the acyl chain preference of PIP5K isoforms for PtdIns4Ps at low substrate concentrations (Fig. 1, *A–C*), where PIP5K isoform γ also shows the strongest preference for SO- over SA-PtdIns4P.

Next we examined whether PIP5Ks exhibit acyl chain preference for activator PA when different species of PtdIns are used as substrates. We used PIP5K isoform γ for these experiments because it has the greatest acyl chain sensitivity to the tested substrates. Interestingly, our data show that there is no significant difference between the degrees of activation by four

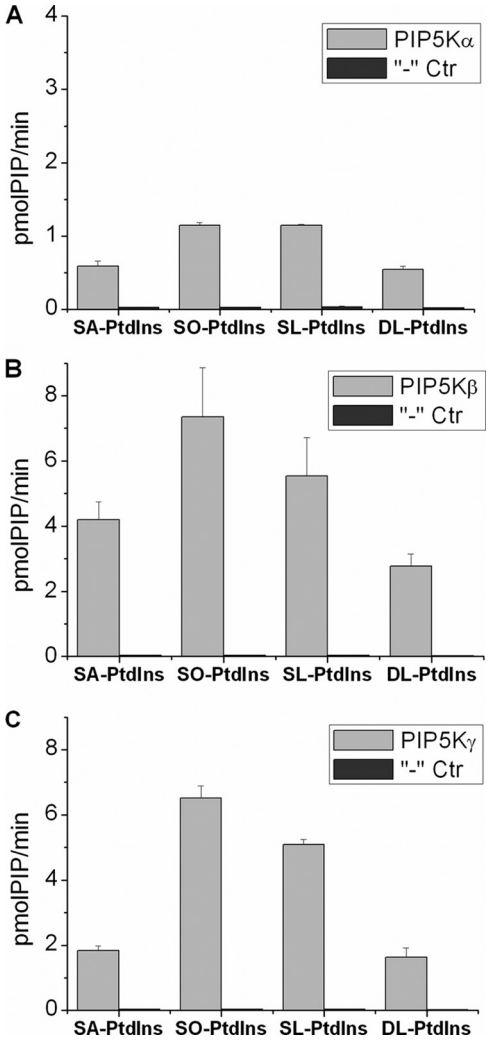

FIGURE 4. **HA-PIP5K isoforms α (*A*), β (*B*), and γ (*C*) show sensitivity to the acyl chain composition of PtdIns substrate.** PIP5K enzymatic activity was measured with 700 μM (equal to C_{eff} = 204 μM) PtdIns. *Error bars*, S.D.

tested PA species with PtdIns as a substrate (Fig. 5). Further, PIP5Kγ is less activated by PAs when the more preferred substrate (SO-PtdIns) is used. It is also surprising that SOPA activates this enzyme when PtdIns is used as a substrate, in contrast to PtdIns4P (Fig. 2).

Thus, PIP5Ks display similar preference for the acyl chain composition of a substrate when either PtdIns or PtdIns4P is used. Nevertheless, there is a remarkable difference in that PIP5K does not show any acyl chain preference for its activator PA when PtdIns is used as a substrate.

Mutants L202I and L210I of PIP5Kα Increase the Extent of Enzyme Activation by PA—Previously, we demonstrated that both L202I and L210I mutations of PIP5Kα decrease the substrate affinity and the enzyme efficiency for SA-PtdIns4P (4). Based on the structure of PIP4KIIβ and protein homology of PIP4K and PIP5K (27, 35), residues Leu-202 and Leu-210 of PIP5Kα are located within the conserved kinase catalytic core and in the putative ATP binding site. To test if the mutations of these residues also affect PA activation of PIP5K, we compared the activation by PA of PIP5Kα WT, L202I, and L210I with three substrates, SA-PtdIns4P, SO-PtdIns4P, and DP-PtdIns4P. Both studied mutations of PIP5Kα significantly increase the extent of enzyme activation by DAPA with all three tested substrates (Fig. 6). However, these mutations do not change the effect of SOPA, which does not activate PIP5Ks with PtdIns4P as a substrate. SAPA, one of the weakest PA activators with PtdIns4P as a substrate, shows only a statistically insignificant tendency toward increased activation for the L202I and L210I mutants of PIP5Kα (Fig. 6).

Study of Interactions between the Monomer Units in the PIP5Ka Dimer—Crystallographic studies of PIP4KIIβ (27) as well as functional analysis of the conserved domains of PIP5Kα (36) suggest that PIP5K forms homodimers. Many proteins are known to function as dimers; nevertheless, the nature of the interaction between the monomers is not clear in most cases. It has been suggested that half-of-sites reactivity may be a common mechanism for tightly associated subunits in homodimers, where both active sites cannot simultaneously be catalytically active, and the monomeric subunit that does not bind substrate plays an enabling role (37–40).

Therefore, to test if PIP5K exhibits similar interactions between the binding sites of the monomeric subunits of the dimer, we compared the activity of PIP5Kα in the state of homodimers and heterodimers, formed between native protein and the kinase-dead mutant of PIP5Kα (D322A).

First, we confirmed that FLAG-PIP5Kα D322A mutant does not exhibit substantial activity with tested substrates SO- and DP-PtdIns4P, compared with the negative control (Fig. 7). Nevertheless, the mutant is significantly activated by the addition of DAPA, especially when SO-PtdIns4P is used as a substrate (Fig. 7). Therefore, it seems that binding of PA to the PA-binding site of PIP5K changes the conformation of the substrate-binding site in a way that allows the phosphorylation of the substrate despite the D322A mutation.

We next tested if there is a cross-talk between the monomeric subunits in a PIP5Kα dimer during PA activation. In the case of the substrate SO-PtdIns4P, the PIP5Kα D322A/D322A homodimer is activated by DAPA more than the wild-type PIP5Kα (WT/WT) homodimer (Fig. 8*A*). However, activation of the heterodimer WT/D322A, rather than being intermediate between the other two constructs, is activated to about the same extent as the D322A/D322A homodimer (Fig. 8*A*). With DP-PtdIns4P substrate, the D322A/D322A homodimer is only weakly activated by DAPA compared with WT/WT (Fig. 8*B*). However, the heterodimeric protein WT/D322A is activated to the same extent as the WT/WT homodimer (Fig. 8*B*). Therefore, these results may indicate that the binding of PA to one of the monomers is sufficient for the activation of dimer activity, similar to the COX-2 inhibition mechanism (37).

We also determined if the interactions between the monomeric subunits in a PIP5Kα dimer are necessary for the enzyme selectivity toward the acyl chains of the substrate. Our results showed that the ratio of enzyme activities with SO-PtdIns4P to DP-PtdIns4P for the WT/D322A heterodimer is similar to the WT/WT homodimer, whereas the D322A/D322A homodimer does not exhibit any substrate preference (Fig. 8*C*). Taken together, these findings suggest that the PIP5Kα dimer may exhibit half-of-sites reactivity, where binding of substrate to the

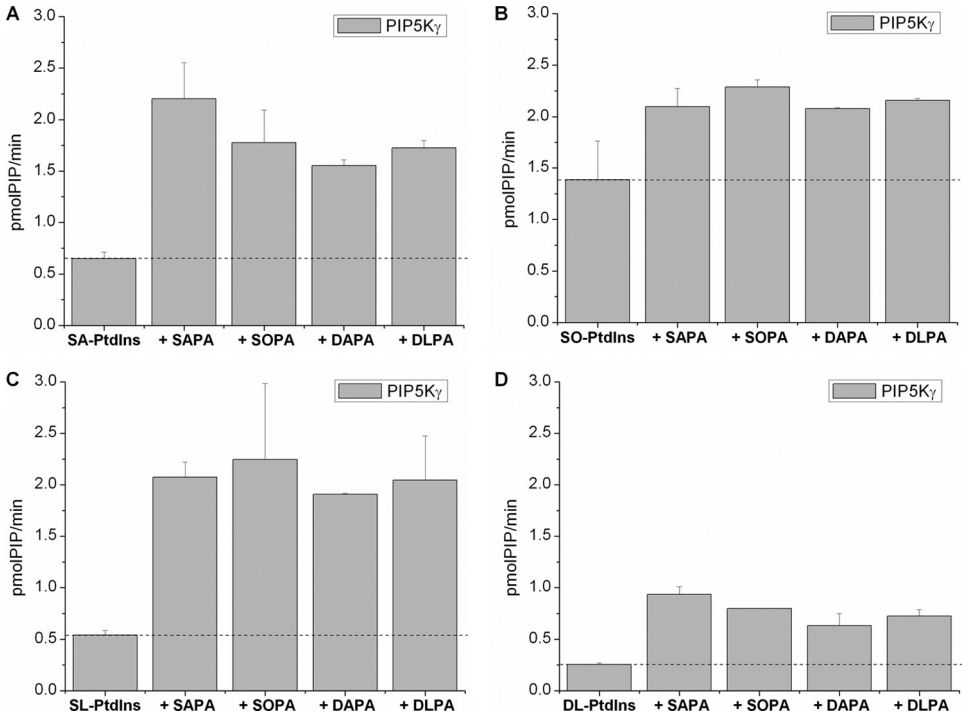

FIGURE 5. **HA-PIP5Kγ does not discriminate between different acyl chains of PA when either SA-PtdIns (*A*), SO-PtdIns (*B*), SL-PtdIns (*C*), or DL-PtdIns (*D*) is used as a substrate.** PIP5K enzymatic activity was measured with 600 μM (equal to C_{eff} = 150 μM) PtdIns and 100 μM (equal to C_{eff} = 4.1 μM) PA. *Error bars*, S.D.

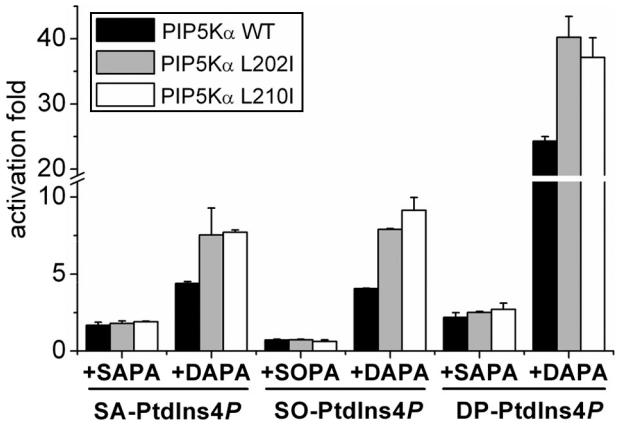

FIGURE 6. **Mutations L202I and L210I of c-Myc-PIP5Kα increase enzyme activation fold by DAPA.** PIP5K enzymatic activity was measured with 10 μM (equal to C_{eff} = 0.06 μM) PtdIns4P and 50 μM (equal to C_{eff} = 1.42 μM) PA. *Error bars*, S.D.

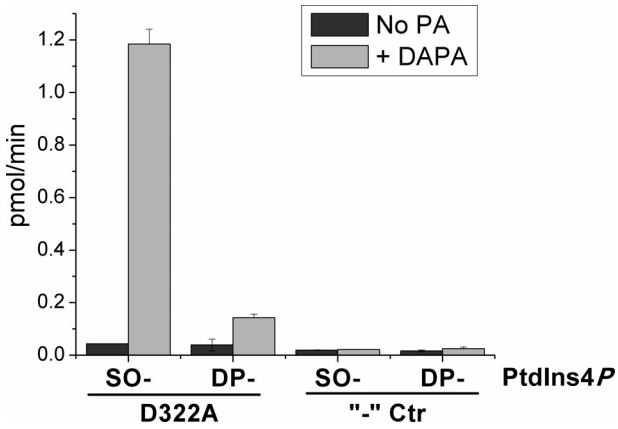

FIGURE 7. **D322A mutant of FLAG-PIP5Kα is activated by DAPA.** PIP5K enzymatic activity was measured with 30 μM (equal to C_{eff} = 0.5 μM) PtdIns4P and 60 μM (equal to C_{eff} = 2 μM) PA. *Error bars*, S.D.

active site of one monomer alters another monomer so that it is unable to bind substrate or activator.

DISCUSSION

PIP5K Sensitivity to the Acyl Chains of Substrate—The acyl chain composition of various lipid classes differs widely (41). Phosphoinositol lipids are mainly polyunsaturated, with 30–80% (depending on the cell type) of total phosphoinositides being the 1-stearoyl-2-arachidonyl species (42–45). 1-Stearoyl-2-oleoyl phosphoinositols were shown to be common species as well, comprising about 11% of total phosphoinositide species in fibroblasts (42). Several lipids serve as secondary messengers, and the proteins that they interact with are greatly affected by their acyl chain composition. For example, PtdIns(4,5)P_2 plays a critical role in endocytosis in synapses by recruiting several essential proteins to the synaptic membranes, including dynamin and the clathrin adaptor proteins (46). At later stages of endocytosis, to decrease the affinity of the clathrin adaptor proteins for the membrane of a synaptic vesicle, PtdIns(4,5)P_2 is dephosphorylated by synaptojanin-1 (47). A previous *in vitro* study showed that the catalytic domain of synaptojanin has a substrate preference for a natural PtdIns(4,5)P_2 compared with DP-PtdIns(4,5)P_2 (48). Therefore, it seems possible that the acyl chain preference of PIP5Ks may facilitate the production of PtdIns(4,5)P_2 species, required for proper downstream cascade in endocytosis.

In addition, it is clear that the PIP5K isoforms function at different cellular locations. For example, PIP5K isoforms modulate endocytosis, exocytosis, and endosomal sorting on dis-

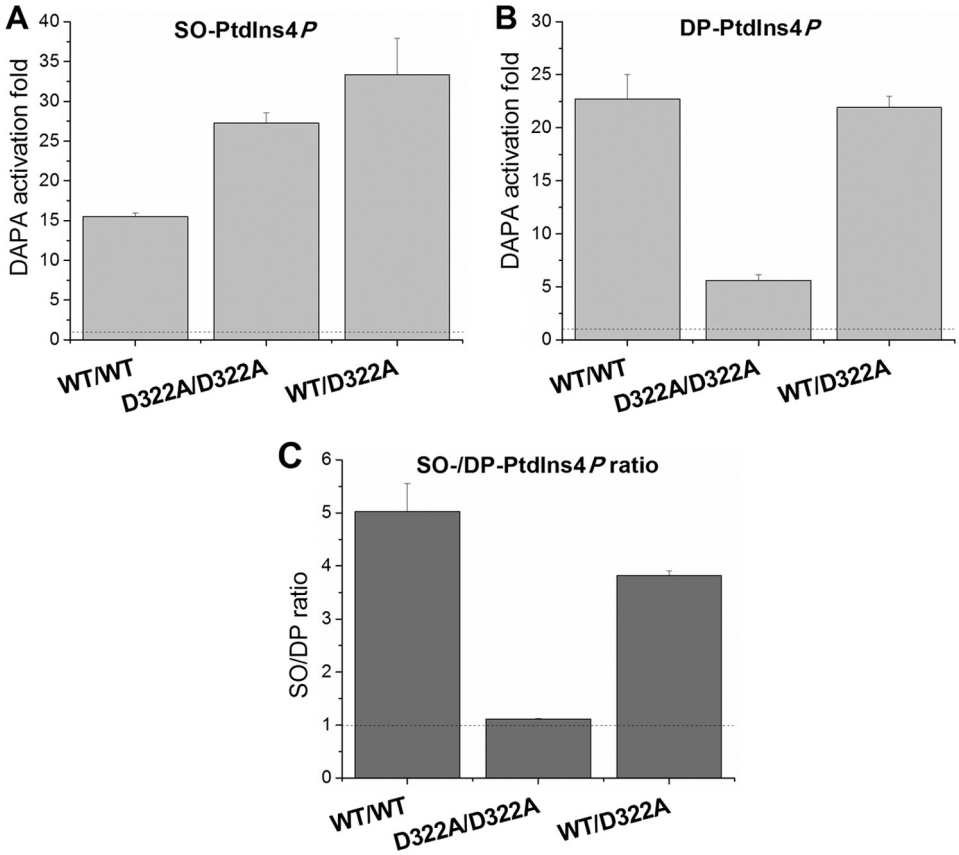

FIGURE 8. *A* and *B*, comparison of DAPA activation of PIP5Kα in a state of WT/WT homodimer, D322A/D322A homodimer, and WT/D322A heterodimer with either SO-PtdIns4P (*A*) or DP-PtdIns4P (*B*) used as substrate. *C*, comparison of enzyme activity ratios with SO- to DP-PtdIns4P used as substrates for PIP5Kα in a state of WT/WT homodimer, D322A/D322A homodimer, and WT/D322A heterodimer. *Error bars*, S.D.

tinct membrane compartments (19, 31). The PIP5Kα also localizes in the nucleus to modulate polyadenylation of mRNA, and this process occurs within undefined lipid complexes (26, 32). Therefore, different isoforms of PIP5K are capable of functioning in highly diverse lipid environments.

PA Activation of PIP5Ks—Activation of PIP5K by PA has been shown to be an important factor in the enzyme regulation (28, 49). Several studies demonstrated that PA generated by PLD, as well as DGKα (50) and DGKζ (13), activates PIP5K *in vivo*, in contrast to PA produced by DGKϵ (50). Therefore, it has been proposed that PA containing monounsaturated and diunsaturated fatty acids activates PIP5K because these PA species are predominantly generated by PLD (51) as well as DGK (predominantly the α and ζ isoforms). These DGK isoforms do not exhibit pronounced acyl chain specificity *in vitro*, phosphorylating different diacylglycerols to a similar extent (52, 53). Our findings indicate that not all monounsaturated and diunsaturated PAs act equally on PIP5Ks. In general, for both SA- and SO-PtdIns4P substrates, there is a noticeable tendency for PAs with both acyl chains unsaturated to be better activators (DAPA, DOPA, and DLPA) than the PA species with a saturated acyl chain. This seems to be an important aspect of PIP5K acyl chain preference for PA because DOPA (18:1/18:1) is a good activator of PIP5K, whereas SOPA (18:0/18:1), having the same lengths of both acyl chains and differing only by one double bond, does not activate the enzyme. Another example is DAPA (20:4/20:4), which is a better activator than AAPA (20:0/20:4) and SAPA (18:0/20:4).

For the physiologically more abundant substrate SA-PtdIns4P, DLPA (18:2/18:2) shows the strongest activation among tested PA species (Fig. 2, *A*–*C*). Surprisingly, when DP-PtdIns4P is used as a substrate, DAPA becomes a very potent activator of all PIP5Ks. Taken together, these findings provide evidence that allosteric activation of the catalytic site of PIP5K by PA is acyl chain-dependent.

PA is also a lipid intermediate of the PtdIns cycle. It is thus possible that different species of PA can result in the feedback activation of the PtdIns cycle. Nevertheless, none of the PIP5K isoforms result in very large feedback activation by the major species of PA in the PtdIns cycle (*i.e.* SAPA). However, DAPA is a good activator with all three of the substrates used and for all three of the isoforms of PIP5K (Fig. 2). In addition to SAPA, DAPA can also be produced efficiently by DGKϵ (53), the isoform of DGK that is closely associated with the PtdIns cycle (54). Thus, there can be a positive feedback activation of the PtdIns cycle by DAPA. However, it should also be noted that PA produced by DGKϵ *in vivo*, SAPA, does not activate PIP5K (50). SAPA will normally be the major product of DGKϵ catalysis. If it did activate PIP5K, it would result in progressively more rapid PtdIns cycling that could be detrimental to the cell. However, it is possible that in particular organs and/or membrane domains or under particular nutritional or pathological states, DAPA

may become the major product of DGKϵ catalysis, leading to this feedback activation of the PtdIns cycle.

Interestingly, PIP5K does not exhibit sensitivity to the acyl chains of PA when PtdIns is used as a substrate (Fig. 5). This may also have physiological relevance because the product of PtdIns conversion by PIP5K is PtdIns5P and not PtdIns(4,5)P_2, which activates PLD. PLD generates PA species that are shown to activate PIP5K, therefore forming a positive feedback loop between these enzymes. In the case when PtdIns is used as a substrate, the PtdIns cycle is not completed, and PLD is not activated. This result also implicates the interplay between the substrate and the activation of PIP5K.

Based on the acyl chain discrimination of PIP5Ks among four tested species of PtdIns and three PtdIns4P substrates, the enzyme preference for the acyl chains of the substrate does not correspond with that of PA. Thus, PIP5Ks have the lowest K_m value for SO-PtdIns4P (Table 2) and exhibit preference for SO-PtdIns among other PtdIns (Fig. 3), whereas SOPA does not activate the enzyme (Fig. 2). On the other hand, DLPA is one of the best activators when SA- or SO-PtdIns4P is used as substrate, whereas DL-PtdIns is not among the preferred substrates (Fig. 5).

The substrate dependence of PIP5K activation by PA was surprising and not anticipated. It is possible that this phenomenon is analogous to the observed substrate-selective inhibition of COX-2, previously described for many drugs (55). The phenomenon arises as a consequence of interactions between monomer units in the COX-2 dimer (37, 56). Both COX-2 and PIP5K form homodimers, so it is possible that PIP5K also exhibits interactions between the binding sites of the monomeric subunits. In the case of COX-2, this leads to substrate-selective inhibition (55, 57), whereas in the case of PIP5K, an analogous process may result in substrate-selective PA activation of the enzyme.

Role of Leu-202 and Leu-210 Residues in PIP5K Activation by PA—Previously, we showed that L202I and L210I mutants of PIP5Kα affect the kinetic parameters of this enzyme for SA-PtdIns4P (4). Here we demonstrate that these mutations also significantly elevate PIP5Kα activation by DAPA but not SOPA or SAPA (Fig. 6). PA binding sites were shown to reside within the C-terminal region of PIP5Kα (residues 239–546 for the murine form of the enzyme). Moreover, this region also mediates interactions with the substrate through the activation and catalytic loops (27, 58). Residues Leu-202 and Leu-210 are located outside these domains but within the conserved kinase catalytic core and proposed ATP binding site. In addition, these residues form part of a segment that resembles the pattern of residues (4, 59) found essential for binding arachidonic acid to lipoxygenase (60). Therefore, our results indicate that mutations of residues Leu-202 and Leu-210 of PIP5Kα enhance the activation of this enzyme by DAPA. This observation is consistent with this segment of the protein being involved with the phosphorylation of polyunsaturated substrates (not necessarily binding; most of the effect is on V_{max}) (4).

Potential Physiological Importance of PIP5K Activation by Dipolyunsaturated PA—It is well recognized that lipid acyl chain composition is important in lipid signaling (61). Most phospholipids of mammalian membranes have a saturated chain at the *sn*-1 position. However, it has been shown that diarachidonoyl-PtdIns is produced in significant quantities when human U937 monocyte-like cells and peripheral blood monocytes are exposed to physiologically relevant concentrations of arachidonic acid (62). Moreover, when the cells are exposed to high concentrations of exogenous arachidonic acid, conditions under which the *de novo* pathway is known to participate in arachidonic acid incorporation into phospholipid (63), DAPA is readily detected, as well as diarachidonoyl glycerol and diarachidonoyl-phosphatidylcholine (62). Although diarachidonoyl species of phospholipids are not abundant in mammalian tissues, they play important roles in cellular functions. For example, diarachidonoyl-phosphatidylcholine is required for the synthesis of anandamide, the endogenous ligand for cannabinoid receptors, which plays crucial roles in the central nervous system and peripheral tissues (64, 65).

Other dipolyenoic lipids have also been shown to occur *in vivo*. Lipid species with two docosahexanoyl chains are known to be present in the retina (66, 67). Furthermore, there is evidence that for cells fed linoleic acid, the levels of DLPA increase to 20% of the total PA in L6 cells and to 8% of the PA in mouse muscle cells (68). DLPA mediates important signaling events by regulating the tyrosine phosphorylation of IRS-1 (68).

PtdIns(4,5)P_2, produced by PIP5Ks, has an essential role in numerous signaling pathways, including actin cytoskeleton remodeling, endocytosis (69), and gene expression (32). PtdIns(4,5)P_2 is the precursor for the second messengers diacylglycerol and inositol triphosphate and also acts directly to modify multiple effectors. The acyl chain composition of PtdIns(4,5)P_2 will be determined in part by the specificity for substrate and activator of PIP5K. This may be an important factor, determining the involvement of different PtdIns(4,5)P_2 species in cellular events.

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