The G protein-coupled receptor kinases (GRKs) phosphorylate agonist occupied G protein-coupled receptors and play an important role in mediating receptor desensitization. The localization of these enzymes to their membrane incorporated substrates is required for their efficient function and appears to be a highly regulated process. In this study we demonstrate that phosphatidylinositol 4,5-bisphosphate (PIP$_2$) enhances GRK5-mediated $\beta$-adrenergic receptor (\$\beta$AR) phosphorylation by directly interacting with this enzyme and facilitating its membrane association. GRK5-mediated phosphorylation of a soluble peptide substrate is unaffected by PIP$_2$, suggesting that the PIP$_2$-enhanced receptor kinase activity arises as a consequence of this membrane localization. The lipid binding site of GRK5 exhibits a high degree of specificity and appears to reside in the amino terminus of this enzyme. Mutation of six basic residues at positions 22, 23, 24, 26, 28, and 29 of GRK5 ablates the ability of this kinase to bind PIP$_2$. This region of the GRK5, which has a similar distribution of basic amino acids to the PIP$_2$ binding site of gelsolin, is highly conserved between members of the GRK4 subfamily (GRK4, GRK5, and GRK6). Indeed, all the members of the GRK4 subfamily exhibit PIP$_2$-dependent receptor kinase activity.

We have shown previously that the membrane association of $\beta$ARK ($\beta$-adrenergic receptor kinase) (GRK2) is mediated, in vitro, by the simultaneous binding of PIP$_2$ and the $\beta$ subunits of heterotrimeric G proteins to the carboxyl-terminal pleckstrin homology domain of this enzyme (Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 11707–11710). Thus, five members of the GRK family bind PIP$_2$ $\beta$ARK (GRK2), $\beta$ARK2 (GRK3), GRK4, GRK5, and GRK6. However, the structure, location, and regulation of the PIP$_2$ binding site distinguishes the $\beta$ARK (GRK2 and GRK3) and GRK4 (GRK4, GRK5, and GRK6) subfamilies.
Interaction of GRK5 with PIP_2

...pholipidPIP_2 inmediatingmembraneassociationofrecent elucidation of the role of the negatively charged phos-
brate association of these GRKs via interaction with the neg-
aturational.GRK5autophosphorylationhasthusbeenproposedto
incorporate receptor substrates (17). Thus, autophosphoryla-
tion, a dramatically impaired ability to phosphorylate membrane-
assayed to examine a potential role for this and other lipids in
in vitro analysis of the Western blot using GRK5 antibodies as described previously (19).

**Purification and Reconstitution of the β2-Adrenergic Receptor—**The human β2-adrenergic receptor (βAR) was expressed and purified from baculovirus-infected S9 cells as described previously (14, 24), Purified receptor was subsequently used to reconstitute plasma membranes by sonication with a microtip sonicator. Purified βAR was reconstituted into artificial membranes as described previously (25). Cos7 cell extracts expressing the polybasic mutants

**EXPERIMENTAL PROCEDURES**

**Materials—**Bovine βARK and GRK5 were overexpressed and puri-
fied from baculovirus-infected S9 cells (18, 19), and G_s subunits were
puriﬁed from bovine brain (20) according to previously published pro-
cedures. Cos7 cell extracts expressing GRK4, -5, and -6 were also utilized as a source of these kinases. GRK4, -5, and -6 were transfected into Cos7 cells using a standard DEAE-dextran procedure (21). Cells were subsequently harvested and lysed, and a soluble cell extract enriched
in these kinases was obtained (7). Rod outer segment mem-
branes, devoid of RK activity, were prepared as described previously (22). Purified lipids and soybean phosphatidylcholine (~20% phosphati-
dylycholine (PC), termed “crude lipid”) were obtained from Sigma.

**Construction of Wild Type and Mutant GRK5 cDNAs—**The cDNA encoding bovine GRK5 (19) was modified to remove all 3′- and 5′-untranslated regions. Using standard polymerase chain reaction tech-
niques as described previously for mutant βARK constructs (7), a new 5′ end containing EcoRI, BglII, and Kozak consensus sequences was added before the start codon, and a new 3′ end containing an XbaI site was inserted immediately after the translational stop codon. The amplified fragment was inserted into the EcoRI/XbaI sites of the expression vectors pcDNAI (Invitrogen) and pRK5 (23). These constructs were used as the wild type GRK5 (GRK5wt) templates for all subsequent molecular manipulations. Mutated GRK5 cDNAs were constructed using standard polymerase chain reaction techniques. The carboxy-ter-
minal polybasic mutant was made using a sense primer starting from the GRK5 Accl site and mutating K547A, K548A, R553A, K556A, and R557A and an antisense primer ending at the 3′ XbaI site. The amplified fragment was digested with Accl/XbaI and ligated with the EcoRI/XbaI fragment of GRK5 into the EcoRI/XbaI sites of the expression plasmid pRK5-GRK5ntyp. The amino-terminal polybasic mutant was made using an antisense primer starting at the GRK5 BulI site and mutating K22A, R23A, K24A, K26A, K28A, and K29A and a sense primer ending at the PsI site. The amplified fragment was digested with BulI/PsiI and inserted into the BulI/PsiI sites of pcDNAI-GRK5NTPB to form pcDNAI-GRK5ntyp. The EcoRI/XbaI fragment
of pcDNA-GRK5NTYP was excised and inserted into the EcoRI/XbaI sites of the psDNA-GRK5NTYP to form psDNA-GRK5NTYP. The autophosphorylation-deficient kinases, the S484A, T485A double mutan-
tant (GRK5ST-AA) and the S484D, T485D double mutant (GRK5ST-DD), were expressed as described previously (17). The amplified fragments were digested with BglII/ XhoI and inserted into the BglII/XhoI sites of the baculovirus transfer vector pVL1392 (PharMingen) to form pVL-GRK5ST-AA and pVL-
GRK5ST-DD. Mutated constructs were verified by double-stranded dye-terminating sequencing of cDNAs using chain termination with Sequenase 2 Polymerase (Amersham Corp./U. S. Biochemical Corp.). The autophosphorylation-deficient kinases, GRK5ST-AA and
GRK5ST-DD were expressed in and purified from baculovirus-infected S9 cells, using the purification procedure described in Ref. 19. In contrast to Cos7 cell extracts expressing the polybasic mutants
GRK5NTYP and GRK5CTYP were used as the source of these kinases. Expression of the mutant GRK5 constructs was confirmed by Western blot analysis using GRK5 antibodies as described previously (19).

**β2-Adrenergic Receptor—**—The β2 (40 nM) reconsti-
tivated in various lipid environments (described in the text and figure legends) was incubated with GRK in 20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 10 mM MgCl_2, 1 mM dithiothreitol containing 60 μM ATP (~6000 cpmpmol) in a total volume of 25 μl. Purified GRK5 or βARK were used at a final concentration of 10 nM. The purified autophosphorylation-
deficient mutants of GRK5 were utilized at equivalent peptide (RRRREEEEEASAA) phosphorylation activities, approximately 15 and 7 nM, respectively, for the GRK5ST-AA, and the GRK5ST-DD. When utilizing Cos7 cell extracts as the source of GRK, equivalent amounts of protein or equivalent rhodopsin kinase activities (as indicated in the figure legend) were utilized in the βAR phosphorylation assay. All assays were performed in the presence of 50 μM (–)isoproterenol, and purified G_s subunits (100 nM) were also included where indicated. Reactions were incubated at 30 °C, stopped by addition of an equal volume of ice-cold buffer (8% SDS, 25 mM Tris, 10% glycerol, 5% mercaptoethanol, 0.003% bromphenol blue), and electrophoresed on 10% SDS-polyacrylamide gels. The dried gels were subjected to autoradiography and phosphorimager (Molecular Dynam-
sis) analysis to determine the pmol of phosphatase transferred to the receptor substrate.

**Pep-dependent Association of GRK5 with Lipid Vesicles—**Vesicles composed of either 100% PC or 95% PC, 5% PIP_2, were incubated in 7 × 20-mM polycarbonate tubes (Beckman) with purified GRK5 (0.5 μg). Incubations were performed for 10 min at 4 °C in phosphate-buffered saline (PBS); the final lipid concentration was 1.7 mg/ml and reaction volume 30 μl. Tubes were subsequently centrifuged at 100,000 rpm (TL-100 rotor) for 15 min at 4 °C. The supernatant was removed and the pellet rinsed once with PBS. The pellet was subsequently resuspended in 15 μl of PBS and transferred to a clean tube. SDS sample-loading buffer was added to the supernatant and pellet fractions, and the samples were electrophoresed on 4–20% gradient polyacrylamide gels (Novex) and subjected to Western blot analysis (ECL, Amersham Corp.) using anti-GRK5 antibodies (19). The distribution of the GRK5 between the pellet (P) and the supernatant (S) was determined by densitometric analysis of the Western blot.

**GRK-mediated Phosphorylation of a Soluble Synthetic Peptide Substrate—**A stock solution of the purified peptide (RRRREEEEEASAA) was prepared and the pH adjusted to 7.2 by the addition of Tris base. GRK-mediated peptide phosphorylation was determined by incubating peptides (1 μM) and the purified kinase (either GRK5 or βARK) in 20 mM Tris-HCl, pH 7.2, 2 mM EDTA, 7.5 mM MgCl_2, and [γ-32P]ATP (~2000 cpm/pmols) at the concentrations indicated. The final reaction volume was 25 μl, and incubations were performed at 30 °C for 15 min. Phosphorylation reactions were linear over this time. Reactions were stopped by spotting onto P-81 phosphocellulose paper (2 × 2 cm squares). Free [γ-32P]ATP was subsequently removed by washing in 75
RESULTS

A role for PIP2 in facilitating βARK-mediated phosphorylation of the βAR has recently been elucidated. Using purified proteins in a reconstituted system, the binding of Goβγ and PIP2 to the βARK PH domain has been shown to be required for membrane association of this enzyme and for βARK-mediated βAR phosphorylation (14). To determine if PIP2 plays a role in facilitating βARK phosphorylation mediated by other members of the GRK family, the βAR reconstituted into vesicles of defined lipid composition was utilized as a substrate for GRK5. GRK5, a member of the GRK4 subfamily, does not possess a PH domain and fails to bind Goβγ (19). This enzyme does contain regions rich in basic amino acids within both its carboxyl and amino termini that potentially represent lipid binding domains.

GRK5-mediated receptor phosphorylation has previously been assessed utilizing as substrates either rhodopsin in rod outer segment membranes or alternatively purified G protein-coupled receptors reconstituted in vesicles composed of heterogeneous and ill-defined lipid mixtures (termed crude lipid environment) (19, 27). As demonstrated in Fig. 1, under these conditions GRK5 mediates agonist-dependent receptor phosphorylation. In marked contrast, the βARK reconstituted in lipid vesicles composed of purified phosphatidylcholine (100% PC) fails to serve as a substrate for this kinase even in the presence of agonist (Fig. 1). These results suggest the presence of essential lipid cofactors for GRK5 in the crude lipid mixture. To examine a potential role for PIP2 in GRK5-mediated βAR phosphorylation, this lipid was incorporated into the receptor containing vesicles. The addition of 5% PIP2 to PC vesicles (95% PC, 5% PIP2) results in a dramatic enhancement of GRK5-mediated agonist-dependent βARK phosphorylation (Fig. 1). PIP2-dependent GRK5 phosphorylation occurs in the absence of Goβγ subunits (Fig. 1) consistent with previous studies demonstrating the Goβγ independence of this enzyme (19).

To determine if the enhanced βARK phosphorylation observed in the presence of PIP2 arises as a consequence of the direct activation of GRK5, GRK5-mediated phosphorylation of a soluble peptide substrate was examined. As shown in Fig. 2, addition of lipid vesicles composed of 100% PC; 95% PC, 5% PIP2 (0.5 µg of PIP2); or 80% PC, 20% PIP2 (2.0 µg of PIP2) had no significant effect on the initial rate of GRK5-mediated peptide phosphorylation. Under these conditions, both the maximal rate of peptide phosphorylation and the concentration of ATP required for half-maximal activation of GRK5 (the Km for ATP) were unaffected by PIP2 addition. These results suggest that the PIP2-dependent enhancement of GRK5-mediated βAR phosphorylation, observed in Fig. 1, arises as a consequence of increased membrane association of GRK5 rather than direct activation of this kinase. That PIP2 indeed promotes vesicle association of GRK5 is shown in Fig. 3. Addition of PIP2 to PC vesicles significantly increases membrane association of GRK5 with 5 ± 5.0% and 70.2 ± 13.2% of the enzyme, respectively, being pelleted in the absence and presence of PIP2 (Fig. 3). PIP2-dependent membrane association of GRK5 was observed in both the presence (Fig. 3) and absence (data not shown) of the βAR substrate. Thus, as with βARK (14), PIP2 appears to enhance GRK5-mediated βARK phosphorylation by promoting the membrane localization of this kinase.

As shown in Fig. 2, low concentrations of PIP2 (0.5–2.0 µg) have no effect on GRK5-mediated peptide phosphorylation and thus no direct effect on GRK5 activity. In contrast, high concentrations of this lipid inhibit GRK5 (Fig. 4A). Addition of 25 µg of 100% PIP2 dramatically and specifically impairs the ability of this kinase to phosphorylate a soluble peptide substrate (Fig. 4A). Similar effects of PIP2 are observed for βARK-mediated peptide phosphorylation (Fig. 4B). Thus, the addition of vesicles containing 2.0 µg of PIP2 has no effect on βARK-mediated peptide phosphorylation (Fig. 4B). High concentrations of vesicles of 100% PIP2 (25 µg), however, dramatically inhibit the maximal rate of βARK-mediated peptide phosphorylation.

These results serve to clarify the somewhat confusing literature concerning the role PIP2 plays in regulating βARK activity. A recent report from our laboratory demonstrates that in vitro the coordinated binding of this lipid and Goβγ to the PH domain of the βARK is required for βARK-mediated βAR phosphorylation (14). In contrast others (28, 29) have reported that PIP2 inhibits βARK activity. The data shown in Fig. 4B provide an explanation for these apparently conflicting results. The differential effects of PIP2 reported by us (14) and by DeBorman et al. (28) and Onorato et al. (29) result from the use of very different PIP2 concentrations. Thus, PIP2-dependent...
βARK-mediated βAR phosphorylation is observed at concentrations of PIP₂ that have no effect on βARK-mediated peptide phosphorylation (Fig. 4B) (14). In contrast the addition of high concentrations of this lipid directly inhibit βARK, resulting in inhibition of βARK-mediated receptor and peptide phosphorylation. The PIP₂ concentrations required to observe inhibition of βARK activity are approximately 12–100-fold higher than those required to observe PIP₂-dependent βARK activity and are equivalent to the inhibitory concentrations of PIP₂ shown in Fig. 4, A and B.

GRK5 undergoes rapid intramolecular phosphorylation to stoichiometries approaching 2.0 mol of P_i/mol of kinase (17, 19). Autophosphorylation has been shown to be stimulated nonspecifically by phospholipids and is proposed to play a role in the membrane localization of GRK5 (17). A role for autophosphorylation in membrane localization is suggested by the observation that, as compared with the wild type enzyme, an autophosphorylation-deficient mutant of GRK5 is specifically impaired in its ability to phosphorylate membrane incorporated receptor substrates (17). PIP₂ may thus potentially facilitate membrane association of GRK5 and thus enhance βAR phosphorylation by stimulating the autophosphorylation of this kinase. To test this hypothesis, the two principal autophosphorylation sites of GRK5 (Ser-484 and Thr-485) were mutated to either alanine (GRK5ST-AA) or aspartic acid residues (GRK5ST-DD) to create, respectively, an autophosphorylation-deficient mutant mimicking the unphosphorylated wild type GRK5 and a mutant in which the autophosphorylation sites are replaced with negatively charged amino acids. The substitution of negatively charged amino acids for phosphorylatable residues has been proposed to mimic the functional effects of phosphorylation (30). The GRK5ST-DD mutant would thus be proposed to be functionally similar to a fully autophosphorylated form of GRK5. These two mutant GRK5s were subsequently assessed for their ability to phosphorylate the βAR in either the presence or absence of PIP₂ (Fig. 5). PIP₂ enhances βAR phosphorylation mediated by either of the GRK5 mutant kinases. Thus, although at equivalent peptide kinase activities the GRK5ST-AA mutant is less active than the GRK5ST-DD mutant phosphorylating the βAR, consistent with previously published observations (17), the activity of both kinases is enhanced by PIP₂ (Fig. 5). The PIP₂-dependent membrane association of GRK5 and enhanced GRK5-mediated βAR phosphorylation thus occurs via an autophosphorylation-independent mechanism.

We investigated the lipid specificity of GRK5. To this end the βAR was reconstituted in purified PC vesicles containing either 3 or 20% of the lipids to be tested (Fig. 6, A and B). As shown in Fig. 6, lipid binding to GRK5 appears highly specific. In vesicles composed of 97% PC and 3% of various lipids, only PIP₂ was capable of promoting GRK5-mediated βAR phosphorylation. Increasing the concentration of lipids in the PC background to 20% increased the extent of GRK5-mediated peptide phosphorylation by 20% (Fig. 6B). At a concentration of 20%, phosphatidylinositol 4-phosphate (PIP) also supported GRK5-mediated βAR phosphorylation, although to a lesser extent than the equivalent concentration of PIP₂ (Fig. 6B). Under these conditions, none of the other lipids tested enhanced GRK5-mediated receptor phosphorylation (Fig. 6). These results support the hypothesis that GRK5 possesses a highly specific binding domain for PIP₂.

To identify which region of GRK5 participates in the binding of PIP₂, mutant GRK5 enzymes were constructed. Since regions rich in basic amino acids have been implicated as potential sites of interaction with negatively charged phospholipids, two such regions, one in the carboxyl and one in the amino terminus of GRK5, were mutated. The amino-terminal basic
amino acid residues Lys-22, Arg-23, Lys-24, Lys-26, Lys-28, and Lys-29 or the carboxyl-terminal basic amino acids Lys-547, Lys-548, Arg-553, Lys-556, and Arg-557 were mutated to alanine residues. These kinases are termed, respectively, GRK5NTPB and GRK5CTPB. The ability of these kinases to phosphorylate the βAR in either the presence or absence of PIP2 is shown in Fig. 7. Extracts derived from Cos7 cells overexpressing these enzymes exhibited very different PIP2-dependent βAR kinase activities (Fig. 7). Thus, although both the wild type enzyme (GRK5) and the carboxyl-terminal GRK5 mutant (GRK5CTPB) exhibited PIP2-dependent βAR phosphorylation, the amino-terminal GRK5 mutant (GRK5NTPB) failed to phosphorylate this receptor substrate. These results suggest that the site of PIP2 interaction may reside within the amino terminus of GRK5 and that the basic amino acids, Lys-22, Arg-23, Lys-24, Lys-26, Lys-28, and Lys-29, play an important role in mediating this interaction. Interestingly, this basic amino-terminal region is highly conserved between members of the GRK4 subfamily (Fig. 8A) suggesting that GRK4 and GRK6 may also be regulated by this lipid. Indeed, cell extracts overexpressing either of these kinases exhibited enhanced βAR phosphorylation in the presence of PIP2 (Fig. 8B). That the PIP2 dependence of the βAR phosphorylation is a regulatory mechanism operating at the level of the GRK rather than at the level of the βAR is supported by the observation that cAMP-dependent protein kinase-mediated βAR phosphorylation is unaffected by the lipid composition of the receptor containing vesicles (Fig. 8B). Thus, all the currently identified members of the GRK4 subfamily appear to bind PIP2 at their amino termini. The binding of this lipid facilitates membrane association of these kinases and promotes phosphorylation of vesicle-incorporated receptor substrates.

**DISCUSSION**

GRK5, a member of the GRK4 subfamily of G protein-coupled receptor kinases, phosphorylates in an agonist-dependent...

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**Fig. 5.** PIP2-dependent GRK5-mediated βAR phosphorylation occurs in the absence of GRK5 autophosphorylation. Purified βAR reconstituted in vesicles composed of either 100% PC or 95% PC, 5% PIP2 were utilized as substrates for wild type GRK5 and two mutants of this kinase. GRK5WT, wild type GRK5. GRK5ST-XX, an autophosphorylation deficient mutant of GRK5 in which Ser-484 and Thr-485 are mutated to alanine residues. GRK5ST-DD, an autophosphorylation mutant of GRK5 in which Ser-484 and Thr-485 are replaced by aspartic acid residues. Phosphorylation reactions were performed at equivalent peptide kinase activities of these enzymes in the presence of 50 μM isoproterenol. The results shown are means ± S.E. from three separate experiments.

**Fig. 6.** The lipid binding site of GRK5 is highly specific for PIP2. Purified βAR reconstituted in 97% PC, 3% (A) or in 80% PC, 20% (B) of the indicated lipids was phosphorylated using purified GRK-5 for 10 min. PA, phosphatidic acid; PI, phosphatidylinositol, PE, phosphatidylethanolamine; MAG, monoacylglycerol (1-monopalmitoyl-rac-glycerol (C16:0)); DAG, diacylglycerol (1, 2-dioleoyl-rac-glycerol (C18:1, [cis]-9); ceramides (type III); GC, galactocerebrosides (type II). The results shown represent the mean values obtained from at least three separate determinations.

**Fig. 7.** The PIP2 binding site of GRK5 is located within the amino terminus of the enzyme. The PIP2-dependent βAR kinase activity of wild type (GRK5) and two mutant GRK5s (GRK5ST-XX and GRK5ST-DD) was assessed. GRK5WT and GRK5CTPB are mutants in which, respectively, the amino-terminal basic amino acids Lys-547, Lys-548, Arg-553, Lys-556, and Arg-557, are mutated to alanine residues. The ability of these enzymes to phosphorylate βAR (1 pmol) reconstituted in vesicles composed of 100% PC or 95% PC, 5% PIP2 (indicated on the figure) was subsequently assessed. Cos7 cell extracts derived from cells expressing either of these three GRKs were used as the source of kinase in these experiments.
Thus, GRK5-mediated lipid is due to PIP2-dependent membrane association of GRK5 in this study and displays PIP2-dependent receptor kinase activity. Interestingly, two of the shorter GRK4 splice variants (GRK4β and -8) lack 32 amino acids at their amino termini and thus lack the postulated PIP2 binding site (16). The potentially differing lipid requirements of the GRK4 splice variants remain to be investigated.

As described previously, PIP2 plays a role in facilitating βARK-mediated βAR phosphorylation (14). Using purified components in a reconstituted system, the simultaneous binding of Gβγ and PIP2 to the PH domain of βARK has been shown to be required for the membrane localization of this kinase and βARK-mediated βAR phosphorylation. Thus, PIP2 plays a role in enhancing the receptor kinase activity of both the βARK and the GRK4 subfamilies of the GRKs. However, the location and structure of the PIP2 binding site as well as the mechanisms regulating the binding of this lipid distinguish the GRK subfamilies. Thus, members of the GRK4 subfamily bind PIP2 via an amino-terminal peptide sequence rich in basic amino acids, a region with similarity to the PIP2 binding site of gelsolin (31). In contrast, the βARK subfamily binds PIP2 via a carboxyl-terminal PH domain (14). Furthermore, PIP2 binds to members of the GRK4 subfamily and promotes receptor kinase activity in the absence of additional ligands. PIP2 binding to members of the βARK subfamily, however, depends on the binding of a second ligand (Gβγ), which increases the apparent affinity of the βARK for PIP2 (14). In contrast to the GRK4 subfamily the presence of PIP2 alone is insufficient to promote βARK-mediated βAR phosphorylation (14). The binding of PIP2 to either GRK subfamily has, however, the similar functional consequence of promoting the membrane association (and thus indirectly the receptor kinase activity) of these enzymes.

The role of the PIP2/βARK interaction has been the subject of some controversy in the literature. Thus although three different laboratories have demonstrated an interaction between the intact βARK and this lipid, different functional consequences arising as a result of this interaction have been reported (14, 28, 29). Research from this laboratory suggests a role for PIP2 in promoting βARK activity (as described above and in Ref. 14) while others have reported PIP2-mediated inhibition of βARK activity (28, 29). These contradictory results can be explained when the different experimental conditions and amounts of PIP2 utilized are considered. As demonstrated in Fig. 4B, the amounts of PIP2 (0.5–2.0 µg) utilized in this and in a previous study from our laboratory (14) have no effect on βARK-mediated phosphorylation of a soluble peptide substrate. PIP2 binding to the βARK thus promotes receptor phosphorylation by facilitating the membrane localization of this enzyme. In contrast, the amounts of PIP2 utilized in the studies of DebBurman et al. (28) and Onorato et al. (29) range between 10 and 50 µg per assay. Under these conditions, PIP2 mediates direct inhibition of the catalytic activity of βARK (Fig. 4B) (29). Thus, high PIP2 concentrations inhibit βARK-mediated βAR phosphorylation. In addition to the amount of PIP2 utilized, one other significant difference distinguishes our studies from those of others, namely the manner in which PIP2 is presented to the GRKs. In the studies of DebBurman et al. (28) and Onorato et al. (29), variable amounts of lipid vesicles composed of 100% PIP2 are directly added to the phosphorylation reactions. In studies from this laboratory, however, the effect of PIP2 is assessed using receptor containing lipid vesicles composed of a mixture of PC and PIP2. Under these conditions the total amount of lipid added per assay remains constant, and the only difference between assay conditions is the variable PIP2 content of the vesicles. PIP2 is thought to represent approximately 0.25–1.0% of total membrane lipid (32, 33). Thus, in our studies the composition of lipid vesicles was varied between 100% PC, 0% PIP2, and 80% PC, 20% PIP2. The lower concentrations of PIP2 utilized by us reflect the use of these, presumably, more physiological membrane environments.

PIP2-mediated membrane association represents only one of a number of potential mechanisms for targeting the GRKs to their appropriate substrates. GRK5 autophosphorylation may also play a role in mediating membrane association of this kinase, since an autophosphorylation mutant of GRK5 has a specifically reduced ability to phosphorylate membrane-incor-
Interaction of GRK5 with PIP\(_2\)

Additional, the association of mutant, GRK5ST-AA, exhibit PIP\(_2\)-dependent that both wild type GRK5 and an autophosphorylation-deficient mutant, GRK5ST-AA, exhibit PIP\(_2\)-dependent GrkARK with specific membrane-localized "anchoring" protein(s) may represent an important mechanism for effecting the membrane localization of this enzyme subfamily (34, 35). The GRKs phosphorylate membrane-incorporated receptor substrates. The membrane localization of these enzymes is thus a prerequisite for efficient function and as such appears to be subject to complex regulatory processes.

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