The β-Adrenergic Receptor Kinase Kinase (GRK2) Is Regulated by Phospholipids*

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The β-adrenergic receptor kinase (βARK) is a member of a growing family of G protein-coupled receptor kinases (GRKs). βARK and other members of the GRK family play a role in the mechanism of agonist-specific desensitization by virtue of their ability to phosphorylate G protein-coupled receptors in an agonist-dependent manner. βARK activation is known to occur following the interaction of the kinase with the agonist-occupied form of the receptor substrate and heterotrimeric G protein βγ subunits. Recently, lipid regulation of GRK2, GRK3, and GRK5 have also been described. Using a mixed micelle assay, GRK2 (βARK1) was found to require phospholipid in order to phosphorylate the β2-adrenergic receptor. As determined with a nonreceptor peptide substrate of βARK, catalytic activity of the kinase increased in the presence of phospholipid without a change in the Km for the peptide. Data obtained with the heterobifunctional cross-linking agent N3-[125I]iodo-4-azidophenylpropionamido-(2-thiopyridyl)-cysteine ([125I]JACTP) suggests that the activation by phospholipid was associated with a conformational change in the kinase. [125I]JACTP incorporation increased 2-fold in the presence of crude phosphatidylcholine, and this increase in [125I]JACTP labeling is completely blocked by the addition of MgATP. Furthermore, proteolytic mapping was consistent with the modification of a distinct site when GRK2 was labeled in the presence of phospholipid. While an acidic phospholipid specificity was demonstrated using the mixed micelle phosphorylation assay, a notable exception was observed with PIP2. In the presence of PIP2, kinase activity as well as [125I]JACTP labeling was inhibited. These data demonstrate the direct regulation of GRK2 activity by phospholipids and supports the hypothesis that this effect is the result of a conformational change within the kinase.

The molecular mechanisms involved in signal transduction of G protein-coupled receptors are best understood in the visual system where rhodopsin serves as the "receptor" for light (1) and the β-adrenergic pathway in which the β-adrenergic receptor (βAR) binds catecholamines (2, 3). A feature common to both model systems as well as many other G protein receptors is the diminished responsiveness with time to a signal of equal intensity. This phenomenon is known as desensitization (4) and exhibits both an agonist-specific and nonspecific pattern. Rapid, agonist-specific desensitization of rhodopsin and the β2-adrenergic receptor (β2AR) occurs in response to the phosphorylation of the receptor by the enzymes rhodopsin kinase and the β-adrenergic receptor kinase (βARK) (5). Rhodopsin kinase and βARK are members of a family known as G protein-coupled receptor kinases (GRKs). A common feature to the GRK family of kinases is multi-site phosphorylation of receptor substrates in response to agonist occupancy (6). The relationship between agonist occupancy and receptor phosphorylation by GRKs is key to the specificity of the desensitization process, while other kinases such as protein kinase A and C play a role in nonspecific or heterologous desensitization. Two possible mechanisms could explain the enhanced phosphorylation of the activated form of the receptor by kinases of the GRK family. First, receptor occupancy may induce a conformational change exposing potential phosphorylation sites previously sequestered from the kinase. Alternatively, interaction of the kinase with the agonist-bound form of the receptor could result in enhanced catalytic activity of the kinase. The bulk of the experimental evidence supports the latter hypothesis (7–9). In addition to the enhanced catalytic activity of GRKs in the presence of agonist-occupied receptor, GRK2 and GRK3 activity is also increased by heterotrimeric G protein βγ subunits (10–13). The potential for finely controlled desensitization by the interplay of receptors and βγ subunits is an exciting possibility given the evidence for dual regulation of GRK2 and GRK3 by these proteins (14).

While G protein-coupled receptors serve as substrates for the kinase after reconstitution into phospholipid vesicles, only recently has specific lipid requirements for GRKs been described. GRK5 was reported to require phospholipid for maximal catalytic activity (15). In this case, phospholipid-stimulated autophosphorylation of GRK5 was necessary for phosphorylation of the β2AR and rhodopsin. In addition, GRK2 and GRK3 were regulated by phospholipids via the interaction with the carboxyl-terminal portion of the kinase known as the plekstrin homology domain (16, 17). In the initial report, the incorporation of negatively charged lipids into phospholipid vesicles resulted in a physical interaction of GRK2 or GRK3 with the vesicle. With the exception of PIP3, this resulted in enhanced phosphorylation of the human m2 muscarinic acetylcholine receptor. The addition of PIP3 resulted in inhibition of phosphorylation of the receptor in a competitive manner with respect to other phospholipids. Purified heterotrimeric G protein βγ subunits

N3-[125I]iodo-4-azidophenylpropionamido-(2-thiopyridyl) cysteine; PIP2, phosphatidylinositol 4,5-bisphosphate; PAGE, polyacrylamide gel electrophoresis.
were able to reverse this inhibition. Furthermore, the lack of additivity suggested a common site of interaction on the kinase for the GRK2 and G protein βγ subunits. This hypothesis was further supported by the finding that two previously characterized G protein βγ subunit binding proteins, phospholipid and glutathione S-transferase-JARK (466–689) fusion protein, prevented the effects of the phospholipids. In a subsequent report, similar effects in terms of PIPγ-enhanced binding of GRK2 to phospholipid vesicles was described. In contrast to the previous manuscript, data are presented that demonstrate increased GRK2 activity when coinubated with both PIPγ and G protein βγ subunits. Additionally, the remaining lipids previ-ously reported to increase kinase activity in the absence of βγ subunits in this case required the addition of G protein βγ subunits to enhance GRK2 activity. The interpretation by these authors was that effective membrane localization of βARK, which enhanced both the rate and extent of phospho-rylation of receptor substrates, required the simultaneous presence of two pleckstrin homology domain ligands.

In this manuscript, we provide evidence of an acidic phos-pholipid requirement of GRK2 based on the phosphorylation of dodecyl maltoside-solubilized receptors and the direct activa-tion of the kinase toward peptide substrates by the addition of various phospholipids. Additionally, lipids that failed to en-hance kinase activity did not increase labeling of GRK2 with the the heterobifunctional reagent [125I]JACTP. Finally, data obtained in the absence of G protein βγ subunits agree with the original report in which PIPγ promotes kinase binding to phospholipid vesicles but inhibits enzymatic activity. Thus, we provide evidence for catalytic activation as well as a confor-mational change in GRK2 following the interaction of the kinase and phospholipid. These data raise the possibility of a third level of regulation of GRK2 activity within the cell and suggest that the mechanism of phospholipid is more complex than simply targeting of the kinase to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—L-sproterenol, alprenol, and all phospholipids were purchased from Sigma. Western blot detection reagents including donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody was obtained from Amersham Corp. The peptide substrate (RRREEEEEESAAA) was synthesized using t-butyloxycarbonyl chemistry with an Applied Biosystems 430A peptide synthesizer. Prior to use, the synthetic peptide was purified by reverse phase high performance liq-uid chromatography using a C-18 column and a 0–50% acetonitrile gradient in 0.1% trifluoroacetic acid/water. [γ-32P]JATP, Na252P, and [125I]iodocyanopindolol were obtained from DuPont NEN. All other reagents were of the highest commercial grade available.

Preparation of βARK—βARK (GRK2) was overexpressed and purified from SF9 cells using the baculovirus expression system as previously detailed (18). Briefly, cells were harvested 48 h after infection by low speed centrifugation. Following homogenization in 20 mM Hapes, pH 7.2, 250 mM NaCl, 5 mM EDTA, 3 mM phenylmethylsulfonyl fluo-ride, and 3 mM benzamidine, a high speed supernatant was prepared. The soluble fraction was diluted and applied to a 2-SP-Sepharose column, which was washed and eluted in a 50–300 mM NaCl linear gradient. Peak activity fractions were pooled, diluted, and loaded on a heparin-Sepharose column. The βARK was eluted from the heparin column using a 100–600 mM NaCl gradient. The peak activity was pooled and made 0.02% final in Triton X-100 and stored at 4°C. Protein concentra-tion was determined by the method of Bradford (19) using purified bovine serum albumin as a standard. Purity of the βARK preparation was determined by SDS-polyacrylamide gel electrophoresis and was routinely >95%.

Preparation of βAR—The hamster βAR was expressed in SF9 cells using the baculovirus system and purified on an alprenol-Sepharose column using a modification of previously described techniques (20, 21). N-Dodecyl β-maltoside (10 mM) in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10 μg/ml each of leupeptin, benzamidine, pepstatin A, and soybean trypsin inhibitor, along with 1 mM phenylmethylsulfonyl fluoride was used to effect solubilization of the receptor from the SF9 cell pellet. Following a high and low salt wash of the alprenol Sepharose column, the βAR was eluted into 50 ml of 1 mM dodecyl maltoside, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA containing 100 μM (-)-alprenolol. The receptor was concentrated to ~2 ml by ultrafiltration on a YM-30 or YM-100 membrane (Amicon) and stored at ~80°C.

For reconstitution studies, the purified receptor was reinserted into phosphatidylincholine vesicles, pelleted by centrifugation, and resus-pended in 20 mM Tris-HCl, pH 7.4, 2 mM EDTA as described previously (22). The concentration of receptor was determined using the β-adre-nergic receptor antagonist [125I]iodocyanopindolol.

For studies in mixed detergent-lipid micelles, the purified receptor was diluted in 1 mM dodecyl maltoside, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA and concentrated on a YM-100 membrane using a centri-cision device (Amicon). Alternatively, receptor purified in detergent underwent liquid organic solvent exchange on a G-50 column equilibrated in 0.5 mM dodecyl maltoside, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA and concentrated on a YM-100 membrane using a centrifuge device. Crude phosphatidylincholine vesicles were produced using a tip sonicator with three 1-min bursts on ice. The desired amount of phospholipid was mixed with dodecyl maltoside-solubilized βAR, from which alprenolol had been described as above, prior to use in the phosphorylation assay.

Phosphorylation of the βAR—Reconstituted βAR (0.03–0.5 pmol) was incubated with GRK2 in a total volume of 25–35 μl containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 7.5 mM MgCl2, 0.1 mM [γ-32P]JATP (200–1,000 cpm/mmol) at 30°C. When indicated, (-)-isoproterenol was included at a final concentration of 10–50 μM. The reaction was stopped by incubation of 50 μl of SAGE stop solution under nitrogen. The reaction was resolved on 9 or 12% polyacrylamide gels (23). Phosphorylated βAR was visualized by autoradiography, and the corresponding bands were excised and counted to determine the extent of phosphate incorporation. Unlike some previous studies with reconstituted receptor, no correction factor for the stoichiometry of receptor phosphorylation was used in these studies.

Phosphorylation of dodecyl maltoside-solubilized βAR was performed in the presence or absence of crude phosphatidylincholine or various purified phospholipids in a buffer of 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 7.5 mM MgCl2, 0.1 mM [γ-32P]JATP (200–1,000 cpm/mmol). The final volume was 50 μl, and the phosphorylation reaction was carried out at 30°C for various times as indicated. The reaction was stopped, and the phosphate incorporation was determined as detailed above.

Phosphorylation of Synthetic Peptides—A stock solution of purified synthetic peptides was prepared, and the pH was adjusted to 7.4 by the addition of Tris base. The peptides were incubated with GRK2 (~80 ng/l) in a buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1 mM [γ-32P]JATP (200–1,000 cpm/mmol), 7.5 mM MgCl2 in a final volume of 25 μl at 30°C. The reaction was stopped by transferring the entire reaction mixture to a 2 × 2-cm square of P-81 paper, followed by six washes in 75 mM phosphoric acid (10 ml/sample). GRK2 activity was defined as the difference in phosphate incorporation in the presence and absence of peptide. Similar results are obtained if the kinase activity was determined in the presence or absence of GRK2. As phosphorylation reactions exhibited a higher blank when performed in the presence of added phospholipid, separate blanks were determined for assays in the absence or presence of additional phospholipid. A nonlinear regression program (Enzfitter, Elsevier-Biosoft, Cambridge, UK) was used to estimate the kinetic parameters.

[125I]JACTP Labeling—[125I]JACTP was synthesized as described pre-viously by Dhanasekaran et al. (24). GRK2 (8 μg or 0.1 nmol) is reacted with an excess (1–200-fold) of [125I]JACTP in dimethylformamide (final concentration of DMF is ~10%). After a 120-min incubation in the dark at 4°C, the reaction was stopped by the addition of SDS (2% final) and 40 mM N-ethylmaleimide. The labeled kinase band was resolved by SDS-PAGE under nonreducing conditions. The GRK2 band was local-ized by autoradiography, excised, and counted. The stoichiometry was calculated after determining the specific activity of the [125I]JACTP preparation (typically 1 Ci/mmol).

Immunodetection of GRK2—Polyacrylamide gels were transferred overnight to nitrocellulose membranes. Immunodetection of GRK2 was performed using a rabbit antibody to purified receptor, raised to provide a peptide (200–1,000 cpm/mmol). Detection of the GRK2 using horseradish peroxidase conjugated donkey anti-rabbit antibody was as described by the manufactur-er (Amersham Corp.).
GRK2 Is Regulated by Phospholipids

A unique feature of the GRK family is the ability to phosphorylate the agonist-occupied form of a variety of G protein-coupled receptors. In order for a receptor to serve as a substrate for the GRK2, the protein is typically purified and reinserted into phospholipid vesicles. If phosphorylation of the receptor in detergent is attempted, no significant incorporation of phosphate is observed. Based on binding data, the receptor exhibits a detergent-solubility requirement for GRKs, only recently have specific lipids required for interaction with GRKs been described (15–17). Fig. 1 demonstrates the agonist-dependent nature of receptor phosphorylation by GRK2. The stoichiometry of phosphorylation is determined by excising the receptor band, quantitating the $^{32}P$ and expressing the data as mol of phosphate/mol of $\beta_{2}$AR.

**RESULTS**

A unique feature of the GRK family is the ability to phosphorylate the agonist-occupied form of a variety of G protein-coupled receptors. In order for a receptor to serve as a substrate for the GRK2, the protein is typically purified and reinserted into phospholipid vesicles. If phosphorylation of the receptor in detergent is attempted, no significant incorporation of phosphate is observed. Based on binding data, the receptor exhibits a detergent-solubility requirement for GRKs, only recently have specific lipids required for interaction with GRKs been described (15–17).

Fig. 1. Phosphorylation of detergent-solubilized $\beta_{2}$-adrenergic receptor by GRK2. $\beta_{2}$AR is expressed in SF9 cells, solubilized in dodecyl maltoside, and purified by affinity chromatography using an alprenolol-Sepharose column. Alprenolol is removed by size-exclusion chromatography on a G-50 column in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 1 mM dodecyl maltoside. The receptor is then concentrated using a Centrioc-100 ultrafiltration device prior to phosphorylation.

Receptor phosphorylation is carried out as described in the text, and the reaction is quenched by the addition of SDS sample buffer. The reaction is resolved by 9% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The phosphorylation reaction is performed in the presence (lanes 1 and 2) or absence (lane 3) of crude phosphatidyldcholine (50 $\mu$g). Isoproterenol (50 $\mu$M) is added (lanes 2 and 3) to demonstrate the agonist-dependent nature of receptor phosphorylation by GRK2. The stoichiometry of phosphorylation is determined by excising the receptor band, quantitating the $^{32}P$ and expressing the data as mol of phosphate/mol of $\beta_{2}$AR.

In order to define the phospholipid specificity of the GRK2 phosphorylation reaction, solubilized $\beta_{2}$AR is added to a variety of neutral, acidic, and basic phospholipids. As shown in Fig. 2, only lipids with a net negative charge including cardiolipin, phosphatidylglycerol, phosphatidic acid, phosphatidylserine, and phosphatidylinositol support the phosphorylation of the $\beta_{2}$AR by GRK2. The addition of crude, but not purified, phosphatidylcholine results in receptor phosphorylation. This suggests that a phospholipid other than phosphatidylcholine is responsible for the activation of GRK2 observed above. Fig. 3 compares the effects of phosphatidylinositol to those of PIP$_2$.

While phosphatidylinositol enhanced receptor phosphorylation by GRK2, there is no significant $\beta_{2}$AR phosphorylation in mixed micelles containing PIP$_2$.

To further investigate the effect of phospholipid on GRK2 activity, a nonreceptor peptide substrate (RRREEEESAAA) previously shown to serve as a $\beta$ARK1 substrate is used (25). The time course of phosphorylation of the peptide by GRK2 is linear for 2 h in the absence or presence of phospholipid (Fig. 4). However, there is a substantial increase in phosphate incorporation observed with the addition of crude phosphatidylcholine to the reaction mixture. The effect of phosphatidylcholine is not due to protection of the kinase from degradation or other nonspecific effects as Western blotting reveals equal amounts of the 80,000 M$_r$ kinase band without evidence of proteolytic cleavage (data not shown).

The kinetic parameters of phosphorylation are determined in the presence of varying amounts of the peptide substrate. As shown in Table I, the effect of phospholipid is to increase the $V_{max}$ of the phosphorylation reaction approximately 3-fold (8.7–25.4 nmol/min·mg of $\beta$ARK) without a change in the $K_m$, for the peptide substrate. In data shown in Table II, phosphatidylinositol increased phosphorylation of the peptide substrate 6-fold while PIP$_2$ decreased GRK2 activity to 30% of the control level.

As GRK5, a member of the GRK family related to $\beta$ARK, has been shown to undergo phospholipid-stimulated autophosphorylation and association with phospholipid vesicles (15), we examine GRK2 to determine if a similar mechanism may be responsible for the phospholipid activation of the kinase. GRK2 does not autophosphorylate to any significant degree in the presence or absence of crude phosphatidyldcholine (Fig. 5). At 1 h, the maximal amount of autophosphorylation is observed with a stoichiometry of 0.1 mol phosphate/mol kinase. A Western blot of GRK2 incubated with vesicles prepared from purified lipids demonstrates a significant amount of immunoreactivity associated with the pellet (Fig. 6). 10–20% of the immunoreactive GRK2 did pellet with phosphatidylinositol, and ~5% pelleted with PIP$_2$. These data stand in contrast to that seen with GRK5 (15) and suggest different mechanisms of lipid activation of the two kinases. Moreover, the demonstration of GRK2 association with vesicles containing either phosphatidylinositol or PIP$_2$ is in agreement with the previously published findings (16, 17).

The heterobifunctional cross-linking reagent, N-3-[125I]iodo-4-azidophenylpropionamido-S-(2-thiopyridyl)cysteine has been used to map the molecular structure of transducin’s $\alpha$ subunit (24). Under mild, nondenaturing conditions, [125I]ACTP derivatizes reduced sulfhydryls to form a mixed disulfide easily cleaved by the addition of excess reducing agents. When GRK2 is incubated for 2 h in the dark with a 100-fold molar excess of [125I]ACTP relative to kinase, there is incorporation of ~1 mol [125I]ACTP/mol kinase. The addition of phospholipid vesicles to the reaction results in a 2-fold enhancement of incorporation to a stoichiometry of 2 mol of [125I]ACTP/mol of $\beta$ARK1 (Fig. 7). The additional [125I]ACTP incorporation observed in the presence of phospholipid vesicles is blocked by the addition of MgATP at concentrations identical to those used in the phosphorylation assay. The effect of MgATP was specific for [125I]ACTP in response to phospholipid as there is no effect observed with [125I]ACTP incorporation in the absence of phos-
pholipid. In all cases, the [125I]ACTP incorporation is sensitive to reducing agents, indicating the presence of a mixed disulfide and not covalent attachment via the azide moiety. When a variety of lipids are examined, only the acidic phospholipids previously shown to enhance GRK2 activity led to an increase in [125I]ACTP labeling of the kinase (Fig. 8). Of note is the observation that PIP_2 not only failed to increase the labeling of GRK2, but decreased [125I]ACTP incorporation to a level below that seen in the basal state. A preliminary mapping experiment demonstrates that the 125I associated with GRK2 resulted in a unique proteolytic map when cleaved with V-8 protease. The appearance of proteolytic bands of 14 and 6 kDa are observed when the kinase is labeled in the presence of the activating lipid phosphatidic acid (Fig. 9). These cleavage products are greatly diminished by co-incubation of lipid and MgATP or with the omission of the phospholipid to the labeling reaction (data not shown).

**DISCUSSION**

Regulation of G protein-coupled receptor function involves the process of desensitization in which a cell exposed to an agonist becomes less sensitive to subsequent stimulation. In the β_2AR-adenyl cyclase system, nonselective, and agonist-specific forms of desensitization occur and appear to be related
to phosphorylation of the receptor (26). Kinases of the GRK family are thought to play a role in rapid, agonist-specific desensitization, as these enzymes phosphorylate the receptor in an agonist-dependent fashion. Several lines of evidence support this proposed role of GRKs in the desensitization process. First, cells that express β2ARs that have had the putative GRK2 phosphorylation sites deleted exhibit delayed desensitization (27). Second, a permeabilized cell system has been used to demonstrate that heparin, a potent inhibitor of GRK2, blocked both agonist-induced receptor phosphorylation and desensitization (28). Third, type-specific antibodies directed toward GRK3 attenuated odorant-induced desensitization in olfactory cells (29, 30). Ishii et al. (31) have shown that GRK3 blocks thrombin signaling when the receptor and kinase are coexpressed in Xenopus oocytes. Finally, overexpression of a GRK2 dominant negative mutant in airway epithelial cells attenuates desensitization of the β2AR (32). At this time, these data are consistent with a role of GRK-mediated receptor phosphorylation in the process of agonist-specific desensitization (27). Second, a permeabilized cell system has been used to demonstrate that heparin, a potent inhibitor of GRK2, blocked both agonist-induced receptor phosphorylation and desensitization (28). Third, type-specific antibodies directed toward GRK3 attenuated odorant-induced desensitization in olfactory cells (29, 30). Ishii et al. (31) have shown that GRK3 blocks thrombin signaling when the receptor and kinase are coexpressed in Xenopus oocytes. Finally, overexpression of a GRK2 dominant negative mutant in airway epithelial cells attenuates desensitization of the β2AR (32). At this time, these data are consistent with a role of GRK-mediated receptor phosphorylation in the process of agonist-specific desensitization.

The agonist-dependent phosphorylation of receptors by GRK2 and other members of the GRK family is a key feature of this class of enzymes. A conformational change in the receptor could expose potential phosphate acceptor sites to the kinase resulting in agonist-dependent phosphorylation of the receptor. However, this does not appear to be the mechanism involved in receptor-GRK interactions (7). Alternatively, the kinase ap-
pears to interact with the agonist-occupied form of the receptor, which primarily results in an increase in the $V_{\text{max}}$ of the enzyme (8, 9). Presumably, a conformational change occurs in GRK2 and other GRKs, which results in enhanced catalytic efficiency. Recently, it has been shown that the peptide mastoparan increases the activity of rhodopsin kinase (8) and a GRK isolated from porcine brain with properties similar to $\beta$ARK1 (13). Since mastoparan activates G proteins by mimicking a structure similar to agonist-occupied receptors (33), a similar mechanism would seem likely in the stimulation of kinase activity.

In addition to the activation of the kinase following the interaction with agonist-occupied receptors, GRKs also interact with membranes via different mechanisms. Photostimulation of rhodopsin results in the association of rhodopsin kinase (8) and a GRK isolated from porcine brain with properties similar to $\beta$ARK1 (13). Since mastoparan activates G proteins by mimicking a structure similar to agonist-occupied receptors (33), a similar mechanism would seem likely in the stimulation of kinase activity.

In the current study, we clearly demonstrate that detergent-solubilized $\beta_2$AR serves as a substrate for GRK2, provided phospholipid is added to the phosphorylation reaction. Under the conditions used in this study, the receptor resides in a mixed detergent-lipid micelle. The concentration of detergent used would not permit the formation of pure lipid vesicles typical of previous reconstitution experiments. Furthermore, the receptor under these conditions does not pellet following a 300,000 $\times$ g centrifugation step adding support to the notion that the receptor is present in mixed micelles. This data would suggest that GRK2 has a phospholipid requirement for phosphorylation of receptor substrates. Using a variety of neutral, acidic, and basic phospholipids, we clearly demonstrate that negatively charged phospholipids, including cardiolipin, phosphatidylglycerol, phosphatidic acid, phosphatidylserine, and phosphatidylinositol, were necessary for phosphorylation of the $\beta_2$AR by GRK2. Previously, purified receptor was first inserted into crude phosphatidylcholine vesicles in order to observe GRK2-dependent phosphorylation. Therefore, we initially performed studies of phospholipid requirements of GRK2 using the same preparation of crude phosphatidylcholine. The fact that crude, but not purified, phosphatidylcholine preparations resulted in kinase activity is consistent with the notion that a phospholipid(s) other than phosphatidylcholine is required by GRK2. Thus, the long recognized requirement for reconstitution of the $\beta_2$AR into phosphatidylcholine vesicles most likely serves to provide a source of negatively charged phospholipid to the phosphorylation reaction.

As mentioned above, the lipid profile demonstrates that phospholipids with a net negative charge at physiologic pH enhance the phosphorylation of the $\beta_2$AR when studied in mixed detergent lipid micelles. A notable exception is the effect of PIP$_2$, as receptor phosphorylation is not observed when this

2 J. J. Onorato, unpublished observation.
phospholipid is included in the phosphorylation assay. Similar data has recently been reported when phosphorylation of the m2 muscarinic acetylcholine receptor was studied in reconstituted lipid vesicles (16). In contrast, others reported thatPIP2-enhanced GRK2 phosphorylation of the β2AR only in the presence of added βγ subunits of heterotrimeric G protein (17). While the stoichiometry of phosphorylation is rather low compared with that previously reported using crude phosphatidylcholine, qualitatively similar results were noted for a variety of lipids tested. In contrast to our findings and that of DebBurman et al. (16), in which <4 mol of phosphate/mol of receptor was achieved in the absence of βγ subunits, a recent manuscript (17) indicated the stoichiometry was <0.5 mol of phosphate/mol of β2AR without the addition of G protein βγ subunits.

The initial step in the mechanism of lipid regulation of GRK2 activity must involve the interaction between lipid and the kinase or the receptor. Evidence of a specific lipid-kinase interaction is provided by the finding that GRK2 becomes associated with vesicles provided they contain negatively charged lipids such as phosphatidylserine, phosphatidylinositol, or PIP2. However, the phosphorylation data presented in this manuscript as well as that previously reported (16) suggests that the effects of lipids are more complex than simply targeting the kinase to the membrane surface. This is evident by the effect of PIP2 to cause membrane association in addition to inhibition of receptor phosphorylation.

In order to test the hypothesis that GRK2 activity is increased by phospholipids, we used a previously characterized peptide substrate of βARK (25). The advantage of the peptide substrate was 2-fold. First, the peptide substrate was designed to bind to ion exchange paper in 75 mM phosphoric acid permitting a large number of phosphorylation reactions necessary to obtain kinetic data. Second, the peptide substrate permits the identification of direct effects of phospholipid upon the kinase in the absence of any possible phospholipid-receptor interactions. The catalytic activity increases 3-fold with respect to the peptide substrate without a change in the Km in the presence of crude phosphatidylcholine. We determined the kinetic parameters using crude phosphatidylcholine as this was the source of lipid that has been used for years in the reconstitution assay. Knowing that the crude preparations were 20% phosphatidylcholine and the results that demonstrate that crude but not purified phosphatidylcholine resulted in receptor phosphorylation in the mixed micelle system, we suspect that other phospholipids were responsible for βARK activity in the reconstitution assay. As further support, we demonstrate that the inclusion of phosphatidylinositol to the peptide assay results in a dramatic enhancement of phosphate incorporation. Moreover, PIP2 inhibited the ability of GRK2 to phosphorylate the synthetic peptide substrate, consistent with our data and that of others (16), in which receptor phosphorylation was studied. In general, peptides are poor substrates for GRKs when compared with reconstituted receptors based on their low affinity for the kinase; however, they provide valuable data as to the mechanism of GRK activation. In this situation, the simplest explanation of the data is that GRK2 is directly activated following interaction with phospholipid. This observation would explain in part the apparent requirement for G protein-coupled receptors to be reconstituted into phospholipid vesicles in order to serve as GRK2 substrates.

Finally, we have used [32P]ACTP as a probe to assess conformational changes that may occur in the kinase. We have observed that GRK2 will incorporate ~1 mol of ACTP/mol of kinase under basal conditions. In the presence of crude phosphatidylcholine, the stoichiometry of [32P]ACTP labeling doubled. We suggest that this represents a sulphydryl group exposed following the interaction of GRK2 and phospholipid. This hypothesis is further supported by three observations. First, the increase in [32P]ACTP incorporation secondary to phospholipid exposure is completely blocked in the presence of MgATP. Second, the V-8 proteolytic map of [32P]ACTP-labeled GRK2 identifies two unique bands in the presence of phospholipid that are diminished under basal conditions or in the presence of MgATP. Finally, lipids, which have been shown to activate GRK2, also increased the [32P]ACTP incorporation. More importantly, PIP2, which binds GRK2 but results in an inhibition of catalytic activity completely abolished any [32P]ACTP labeling, indicating what appears to be the site labeled in the absence of lipids. At this time, our working hypothesis is that the sulphydryl group(s) exposed following phospholipid interaction with GRK2 is near the ATP binding site and/or catalytic groove of the kinase and protected from ACTP labeling by the binding of MgATP. Furthermore, our ability to label this additional site serves as a probe of a putative conformational change, which occurs as the kinase is activated by regulatory lipids.

Data presented in this manuscript provide the first direct evidence to support the direct regulation of GRK2 by lipid. The effect on catalytic activity, in addition to the membrane localization, which occurs via the interaction between various lipids and the pleckstrin homology domain of GRK2 and GRK3, have clear implications as to the regulation of the kinase. Given the apparent association of several members of the GRK family with phospholipid membranes, it is tempting to speculate that many of the GRKs require phospholipid for maximal catalytic activity. While several different types of interaction between various GRKs and phospholipid membranes have been described, it will prove valuable to test whether a common molecular mechanism exists among this family of kinases. We are currently mapping the sites of ACTP incorporation in GRK2 to permit such a study. Additional studies are ongoing to define specific phospholipid interactions with βARK and extend the current studies to other members of the GRK family.

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