THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 18, Issue of May 4, pp. 15192–15199, 2001
Printed in U.S.A.

Regulation of Membrane Targeting of the G Protein-coupled Receptor Kinase 2 by Protein Kinase A and Its Anchoring Protein AKAP79*

Received for publication, October 5, 2000, and in revised form, January 18, 2001
Published, JBC Papers in Press, January 22, 2001, DOI 10.1074/jbc.M009130200

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The β2 adrenergic receptor (β2AR) undergoes desensitization by a process involving its phosphorylation by both protein kinase A (PKA) and G protein-coupled receptor kinases (GRKs). The protein kinase A-anchoring protein AKAP79 influences β2AR phosphorylation by complexing PKA with the receptor at the membrane. Here we show that AKAP79 also regulates the ability of GRK2 to phosphorylate agonist-occupied receptors. In human embryonic kidney 293 cells, overexpression of AKAP79 enhances agonist-induced phosphorylation of both the β2AR and a mutant of the receptor that cannot be phosphorylated by PKA (β2AR/PKA−). Mutants of AKAP79 that do not bind PKA or target to the β2AR markedly inhibit phosphorylation of β2AR/PKA−. We show that PKA directly phosphorylates GRK2 on serine 685. This modification increases Gβγ subunit binding to GRK2 and thus enhances the ability of the kinase to translocate to the membrane and phosphorylate the receptor. Abrogation of the phosphorylation of serine 685 on GRK2 by mutagenesis (S685A) or by expression of a dominant negative AKAP79 mutant reduces GRK2-mediated translocation to β2AR and phosphorylation of agonist-occupied β2AR, thus reducing subsequent receptor internalization. Agonist-stimulated PKA-mediated phosphorylation of GRK2 may represent a mechanism for enhancing receptor phosphorylation and desensitization.

Hormonal signaling through G protein-coupled receptors (GPCRs)[1] is attenuated during prolonged exposure to agonist, a process known as desensitization (1). One of the initial events in this multistep process is the phosphorylation of agonist-occupied receptor molecules. Two families of kinases are responsible for the phosphorylation of GPCRs, the second messenger-activated kinases (PKA and PKC) and the GPCR kinases (GRKs 1–7) (2–8). Although the second messenger-activated kinases are capable of phosphorylating receptors in the absence or presence of agonist, the GRKs specifically phosphorylate only agonist-occupied receptors (5, 9). Moreover, phosphorylation by GRKs leads to the recruitment of the arrestin proteins to the phosphorylated receptors, preventing further coupling to G proteins (1). In the case of the prototypic β2 adrenergic receptor (β2AR), agonist stimulation leads to the recruitment of cytosolic GRK2 to the plasma membrane where it binds to and phosphorylates the receptor. The mechanisms by which different GRKs are recruited to the receptor varies, but for both GRK2 and GRK3, recruitment is achieved through the binding of phospholipids and Gβγ subunits to the COOH-terminal pleckstrin homology domain of the kinase (10–16). As the recruitment requires the presence of free Gβγ subunits, the binding of the kinase to the receptor only occurs at times when the receptor is signaling through coupling to G proteins. In this manner, the kinase is only delivered at times when there are agonist-occupied receptor substrates for it to phosphorylate.

The second messenger-activated kinases are constitutively localized to subcellular structures via their interactions with anchoring proteins (17). In the case of PKA, its localization is achieved through a family of A kinase-anchoring proteins (AKAPs) that were originally identified by their ability to interact with PKA regulatory subunits II (18). The AKAPs are responsible for the localization of PKA to particular subcellular compartments, including the plasma membrane, mitochondria, post-synaptic densities, and centrosomes (19–27). Some of the AKAPs have been shown to exist in complexes containing a number of other signaling molecules, including the *N*-methyl-D-aspartate receptor, ion channels, GPCRs, and protein phosphatases (27–33). These discoveries have led to the hypothesis that AKAPs may act as nodes at which cross-talk between different signaling events may be coordinated (34). One family member, AKAP79 (human AKAP79, rodent AKAP150, and bovine AKAP75), binds not only to PKA but also to PKC and the protein phosphatase 2B (20, 34–37). AKAP79 has also been shown to be responsible for the association of PKA with the plasma membrane and with integral membrane proteins including the β2AR (27). It has been demonstrated that the overexpression of AKAP79 with the β2AR enhances receptor phosphorylation and, furthermore, that mutants of AKAP79, which fail to bind to the β2AR or to PKA, are effective at

* This work was supported in part by National Institutes of Health Grants HL16037 (to R. J. L) and GM48231 (to J. D. S). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; AKAP, A kinase-anchoring protein; PKA, protein kinase A; PKC, protein kinase C; β2AR, β2 adrenergic receptor; HEK, human embryonic kidney; GRK, G protein-coupled receptor kinase; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; Erk1/Erk2, extracellular signal-regulated kinase 1 and/or 2.

reducing phosphorylation. In this manner, AKAP79 may be acting as a scaffold which coordinates the events involved in receptor desensitization. To investigate this novel function of AKAP79 further, we set out to test whether another event involved in β2AR desensitization, *i.e.* receptor phosphorylation by GRK2, is modulated by PKA scaffolding.

EXPERIMENTAL PROCEDURES

Materials

GRK2 was purified from baculovirus-infected Sf9 cells as described previously (38). Bovine GRK2ct (residues 467–689) was expressed as a glutathione *S*-transferase (GST) fusion protein in bacteria and purified as described previously (13). Purification of rod outer segment membranes (39), Gβγ subunits, (40) and tubulin (41) was described previously. AKAP79, AKAP79pro, and AKAP79$^{108-427}$ mammalian expression constructs were described previously (27, 37). Mammalian expression constructs of FLAG epitope-tagged β2 adrenergic receptor (β2AR) and β2AR/PKA− mutant receptor (PKA phosphorylation sites serines 261, 262, 345, and 346 all mutated to alanines) were described previously (42). The PKA catalytic subunit was purchased from Promega, and the anti-Gβ antibody was from Perkin Elmer Life Sciences. The anti-GRK2 monoclonal and AKAP79 polyclonal antibodies were described previously (20, 42). M2 anti-FLAG antibody conjugated to Sepharose beads and M2 antibody were from Sigma. Unless otherwise stated, the chemicals were from Sigma.

Plasmid Constructs

Construction of the pcDNA3-Bovine GRK2 Mutant S685A—pcDNA1-bovine GRK2 (43) was restriction digested with *Hin*dIII and *Xba*I, and the insert was ligated into the pcDNA3 vector (Invitrogen). A single point mutation changing serine 685 to alanine was introduced by the polymerase chain reaction using the primers 5′-TCCCCAACCGC-CTCGAGTGGC-3′ and 5′-CTAGTCTAGATCAGAGGCCGTTGGCG-GCGCCGCGC-3′. The polymerase chain reaction product was restriction digested with *Xho*I and *Xba*I and used to replace the equivalent fragment in the pcDNA3 construct. The sequence and orientation of the clone were confirmed by automated DNA sequence analysis.

Construction of a COOH-terminal Truncated GRK2Δ19 Baculovirus Plasmid—The *Rsr*II/*Bam*HI restriction fragment from a GRK2Δ19 construct (construct 2 in Ref. 13 encoding full-length GRK2 with a stop codon inserted at codon 671) was used to replace the equivalent fragment in pVL1392-GRK2(S670A) (44). The orientation and sequence of the clone were confirmed by automated DNA sequence analysis. Purification of the expressed protein from Sf9 cells was identical as that described for full-length GRK2 (38).

Methods

Cell culture, Immunoprecipitations, and Immunoblotting—HEK293 cells were grown at 37 °C in minimal essential medium containing 10% fetal bovine serum and 1× penicillin/streptomycin (Life Technologies, Inc.) under 5% CO_2. Cells at 60% confluence were transfected with up to 5 μg of plasmid DNA and 15 μl of Fugene 6 (Roche Molecular Biochemicals). Two days after transfection, cells were lysed in radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% v/v Nonidet P-40, 0.5% w/v sodium deoxycholate, 10 mM NaF, 10 mM Na_2-pyrophosphate, 0.1% w/v SDS, 5 μg/ml aprotinin, 150 μg/ml benzamidine, 5 μg/ml leupeptin, 4 μg/ml pepstatin, and 20 μg/ml phenylmethylsulfonyl fluoride). If cross-linking of proteins was necessary before immunoprecipitation, cells were incubated at room temperature for 20 min in phosphate-buffered saline containing 10 mM HEPES, pH 7.4, and 1 mg/ml dithiobis(succinimidyl propionate) before lysis in radioimmune precipitation buffer (45). After removal of insoluble cell debris by centrifugation, protein concentrations were equalized

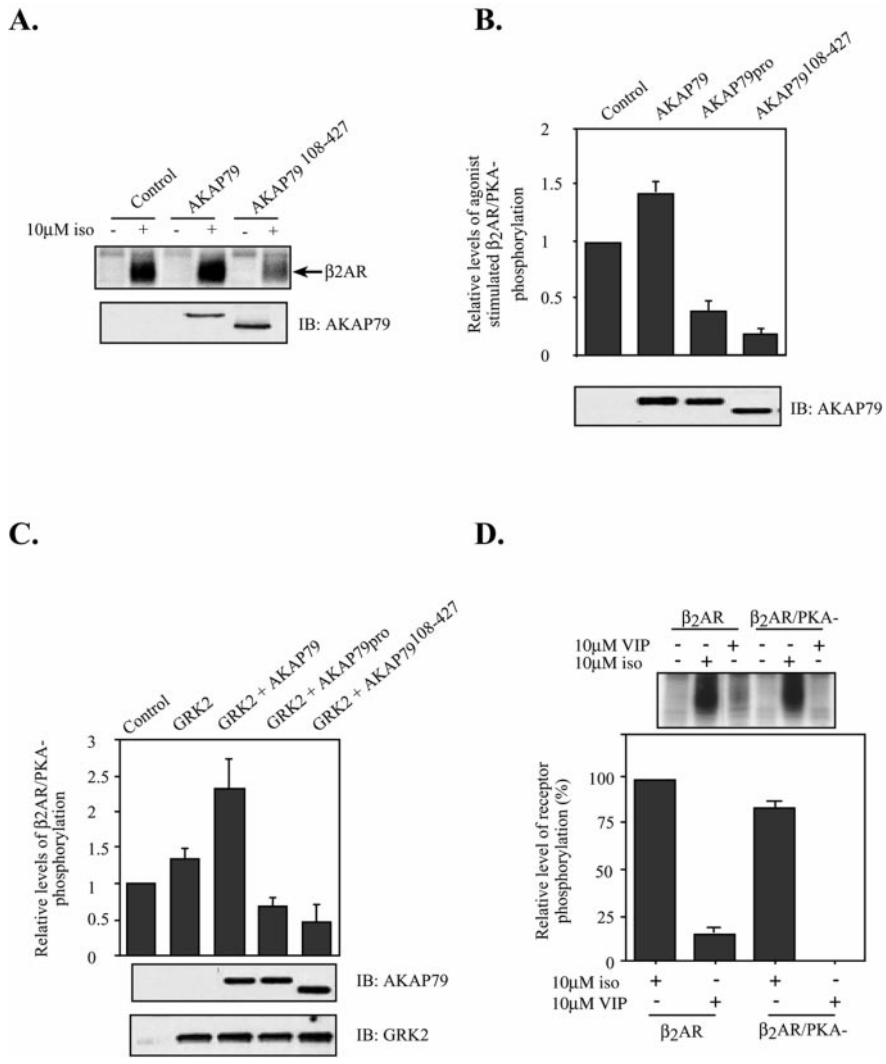

FIG. 1. **Effects of PKA anchoring on β2AR and β2AR/PKA− phosphorylation.** *A,* agonist-stimulated β2AR phosphorylation is regulated by AKAP79. HEK293 cells overexpressing FLAG-β2AR were transiently transfected with AKAP79 or the mutant AKAP79$^{108-427}$ and incubated in [^{32}P]orthophosphate. Cells were stimulated with 10 μM isoproterenol (*iso*) for 5 min, and FLAG-β2AR was immunoprecipitated and resolved by SDS-PAGE. *Upper panel* shows a representative autoradiogram ($n = 4$) with basal (−) and isoproterenol-stimulated (+) β2AR phosphorylation in transfected cells as indicated. AKAP79 and AKAP79$^{108-427}$ expression was detected in cell lysates by immunoblotting (*IB, lower panel*). *B,* agonist-stimulated β2AR/PKA− phosphorylation is regulated by AKAP79. HEK293 cells expressing FLAG-β2AR/PKA− were transiently transfected with AKAP79, AKAP79pro, or AKAP79$^{108-427}$, and isoproterenol-stimulated phosphorylation of β2AR/PKA− was measured as described earlier. The *graph* shows the mean levels of phosphorylation of β2AR/PKA− relative to control transfected cells. AKAP79, AKAP79pro, and AKAP79$^{108-427}$ expression were detected in cell lysates by immunoblotting (*lower panel*). *C,* AKAP79 regulates β2AR/PKA− phosphorylation by GRK2. HEK293 cells expressing β2AR/PKA− were transiently transfected with GRK2, AKAP79, AKAP79pro, and AKAP79$^{108-427}$ as indicated. Isoproterenol-stimulated phosphorylation of β2AR/PKA− was measured as described earlier. Expression of AKAP79, its mutants, and GRK2 was confirmed by immunoblotting (*lower panels*). *D,* β2AR/PKA− cannot be phosphorylated by PKA. FLAG-β2AR or FLAG-β2AR/PKA− was transfected into HEK293 cells. Cells were subsequently stimulated with 10 μM isoproterenol or 10 μM vasoactive intestinal peptide (*VIP*) for 5 min, and ^{32}P incorporation was measured. The *graphs* show the mean ± S.E. of three experiments.

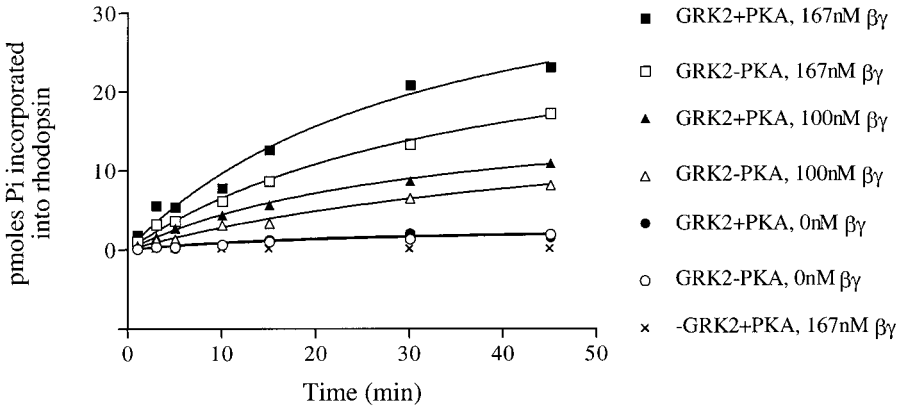

FIG. 2. **Effect of PKA-mediated phosphorylation of GRK2 on rhodopsin phosphorylation.** Twenty-five nanograms of GRK2 phosphorylated by PKA (*closed symbols*) or not (*open symbols*) was used to phosphorylate 2 μg of rhodopsin in the presence of 0, 100, or 167 nM G$\beta\gamma$. Phosphorylation reactions were performed at 30 °C for the indicated times. Reactions were stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer, resolved by SDS-PAGE, and radioactive bands were quantified using a PhosphoImager. The results shown are representative of three experiments.

in all samples, and FLAG epitope-tagged proteins were immunoprecipitated for 15 h with 40 μl of a 1:1 slurry of M2 anti-FLAG antibody covalently coupled to Sepharose beads. The beads were washed four times with radioimmune precipitation buffer, and bound proteins were eluted in 50 μl of 2× SDS-PAGE sample buffer (100 mM Tris, pH 7.2, 4% w/v SDS, 200 mM dithiothreitol, 20% v/v glycerol, 20 μg/ml bromphenol blue with 5% v/v β-mercaptoethanol for cross-linked samples) for 10 min at 95 °C. Samples were resolved on 10% or 4–20% polyacrylamide gels (Novex) and transferred to nitrocellulose filters for immunoblotting. Filters were blocked with 5% w/v fat-free milk powder in Tris-buffered saline with Tween 20 (20 mM Tris, pH 7.4, 500 mM NaCl, 0.1% v/v Tween 20) and incubated overnight at 4 °C with appropriate primary antiserum. After thorough washing in Tris-buffered saline with Tween 20, filters were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or mouse secondary antibody (Amersham Pharmacia Biotech), washed again with Tris-buffered saline with Tween 20, immersed in ECL reagent (Amersham Pharmacia Biotech), and exposed to x-ray film.

Receptor Sequestration Assay—COS7 cells expressing FLAG-β2AR/PKA− alone or coexpressing AKAP79 with GRK2 or GRK2S685A were stimulated with 10 μM isoproterenol for 30 min. Agonist-induced receptor internalization was measured as the loss of cell surface FLAG epitopes available for M2 antibody binding by detection of a fluorescently labeled secondary antibody as described previously (46).

Receptor Phosphorylation Assay—Receptor phosphorylation was assessed after the labeling of the intracellular ATP pool of HEK293 cells stably transfected with FLAG-β2AR or FLAG-β2AR/PKA− with [^{32}P]orthophosphate (PerkinElmer Life Sciences) as described previously (43). Cells were labeled for 1 h at 37 °C in phosphate-free Dulbecco's modified Eagle's medium containing 10 mM HEPES, pH 7.4, 100 μCi/ml [^{32}P]orthophosphate, and 1 μg/ml microcystin L-R (Calbiochem). Cells were stimulated with 10 μM isoproterenol for 5 min, washed twice with ice-cold phosphate-buffered saline buffer, solubilized in 750 μl of radioimmune precipitation buffer, and equivalent protein amounts were subjected to immunoprecipitation. Samples were resolved on 10% polyacrylamide gels and dried under vacuum. Radioactive bands were visualized and quantified using a PhosphorImager (Molecular Dynamics) and by exposure to x-ray film. The levels of receptor expression were measured by flow cytometry by detecting cell surface-bound anti-epitope tag M2 antibodies and fluorescein-conjugated secondary antibodies (46). All phosphorylation levels were normalized to receptor expression and are shown as stimulated-basal values.

In Vitro PKA-mediated Phosphorylation of Full-length and Fragments of GRK2—One microgram of GRK2 or GRK2Δ19 purified from baculovirus-infected Sf9 cells or GRK2ct purified by cleavage with thrombin from bacterially expressed GST fusion protein (13) was incubated with 1 unit of PKA catalytic subunit (Promega) in phosphorylation reaction buffer containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 60 μM [γ^{32}P]ATP (~1000 cpm/pmol), 1 μg/ml phospholipids (crude preparation containing 20% w/v L-α-phosphatidylcholine, Sigma), and 0.8 μM G$\beta\gamma$ subunits. After 30 min of incubation at 30 °C, reactions were stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer. Samples were boiled for 10 min and resolved on 4–20% gradient polyacrylamide gels. Radioactive bands in dried gels were visualized and quantified using a PhosphorImager and by exposure to x-ray film.

GRK2-mediated Phosphorylation of Protein Substrates—*In vitro* phosphorylation of rhodopsin, tubulin, and the peptide substrate RRRREEEEESAAA by GRK2 was performed as described previously (47). The reactions used 25 ng of GRK2 to phosphorylate 2 μg of rhodopsin or 100 ng of GRK2 to phosphorylate either 0.2 μg of tubulin or 1 mM peptide substrate.

In Vitro Binding of G$\beta\gamma$ to GST-GRK2ct—One microgram of GST-GRK2ct (residues 467–689 of bovine GRK2) bound to glutathione-conjugated Sepharose 4B beads (Calbiochem) was incubated with or without PKA as described above. After 30 min of incubation at 30 °C, beads were washed extensively with phosphate-buffered saline containing 0.01% v/v lubrol to remove all the PKA and ATP. Four micrograms of G$\beta\gamma$ from bovine brain was then added and incubated at 4 °C for 2 h. After extensive washes with phosphate-buffered saline containing 0.01% lubrol, 2× sample buffer was added, and proteins were eluted by boiling for 10 min. Samples were resolved on 4–20% polyacrylamide gels, transferred to nitrocellulose filters, and immunoblotted with an antibody recognizing Gβ subunits.

RESULTS

It has been shown previously that the protein kinase A-anchoring protein AKAP79 can directly interact with and regulate the phosphorylation of the β2AR (27). In HEK293 cells overexpressing FLAG epitope-tagged β2AR, the coexpression of AKAP79 increases β2AR phosphorylation by 40%, whereas the coexpression of the AKAP79 mutant AKAP79$^{108-427}$, which does not bind to the receptor or localize PKA to the membrane, impairs receptor phosphorylation by 50% (Fig. 1*A*). To determine whether this enhancement of β2AR phosphorylation is a result of AKAP79 directly facilitating phosphorylation of the receptor by PKA, agonist-induced phosphorylation of a FLAG epitope-tagged β2AR mutant lacking all PKA phosphorylation sites (β2AR/PKA−) was measured. Surprisingly, the coexpression of wild type AKAP79 enhances agonist-induced phosphorylation of β2AR/PKA− by 40% (Fig. 1*B*). In contrast, cells transfected with either of two AKAP79 mutants that are unable to target PKA to the plasma membrane, AKAPpro and AKAP$^{108-427}$, exhibit a 55–70% decrease in β2AR/PKA− phosphorylation. AKAP79pro is targeted to the plasma membrane and can bind to β2AR but is unable to bind to the PKA regulatory subunit, whereas AKAP79$^{108-427}$ binds to PKA but lacks the receptor and membrane-targeting domain (27, 37). Although GRK2 and AKAP79 do not interact directly (data not shown), when GRK2 and AKAP79 are overexpressed together a 2.3-fold increase in β2AR/PKA− phosphorylation is observed compared with a 1.4-fold increase by overexpression of GRK2 alone (Fig. 1*C*). Overexpression of either of the two AKAP79 mutants with GRK2 inhibits receptor phosphorylation by 40–60% compared with control cells expressing only endogenous levels of GRK2 (Fig. 1*C*).

These results suggest that AKAP79 does not affect β2AR phosphorylation by simply enhancing direct receptor phosphorylation by PKA. Rather, they suggest that AKAP79 enhances GRK2-mediated phosphorylation of the β2AR. To ensure that PKA is not capable of phosphorylating the β2AR/PKA− mutant, the phosphorylation of the wild type receptor and β2AR/

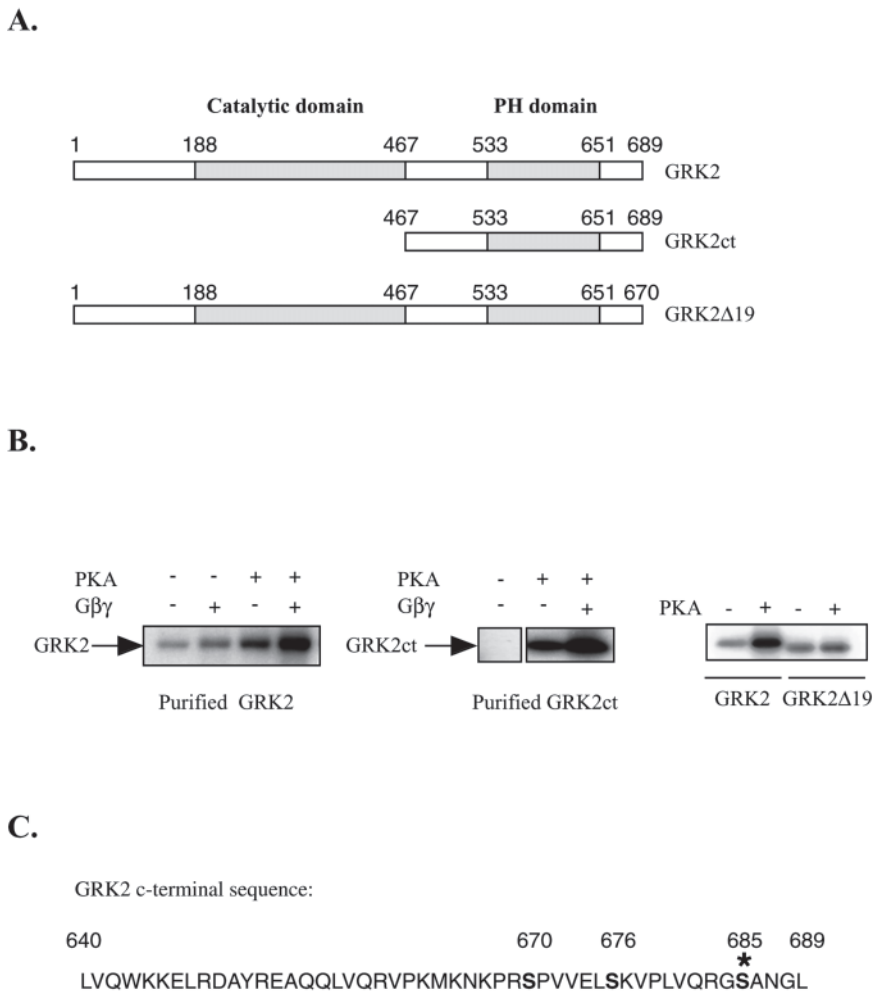

FIG. 3. **Identification of GRK2 as a PKA substrate.** *A, schematic* representation of GRK2 fragments used as substrates in PKA phosphorylation assays. Full-length GRK2 (residues 1–689) contains a central catalytic domain (residues 188–467) and a COOH-terminal pleckstrin homology domain (*PH domain*, residues 533–651) . The GRK2ct fragment (residues 467–689) contains only the pleckstrin homology domain, and GRK2Δ19 is identical to full-length GRK2 but with the last 19 residues deleted. *B,* purified wild type and fragments of GRK2 were phosphorylated by PKA for 30 min at 30 °C in the presence of [γ-^{32}P]ATP, phospholipids, and with or without G$\beta\gamma$ subunits added. Samples were resolved by SDS-PAGE. Shown are representative autoradiograms from three experiments. *C,* sequence of the last 49 amino acids of GRK2 with potential serine phosphorylation sites shown in *bold* and the PKA phosphorylation site, serine 685, marked with an *asterisk*.

PKA− was compared following activation of PKA by stimulation of a coexpressed Gs-coupled receptor (Fig. 1*D*). In the absence of its ligand, the β2AR can be phosphorylated by PKA but not by the GRKs as they specifically phosphorylate only agonist-occupied receptors. Stimulation of endogenous vasoactive intestinal peptide receptors leads to the phosphorylation of wild type β2AR to 20% of the level observed with isoproterenol stimulation (Fig. 1*D*). This level is consistent with previous reports for PKA phosphorylation of the β2AR in HEK293 cells (48). The β2AR/PKA− mutant, however, shows no vasoactive intestinal peptide-induced phosphorylation (Fig. 1*D*), confirming that the removal of the PKA phosphorylation sites renders the receptor incapable of being further phosphorylated by PKA. Taken together, the regulation of β2AR/PKA− phosphorylation by wild type and mutants of AKAP79 indicates that AKAP79 may indirectly regulate GRK2 activity by a mechanism requiring the anchoring of PKA to the plasma membrane.

To further test the ability of PKA to affect GRK2 activity against receptor substrates, we measured the effect of PKA on GRK2-mediated phosphorylation of rhodopsin *in vitro*. Incubation with PKA and 167 nM G$\beta\gamma$ subunits or with GRK2 alone results in little or no phosphorylation of rhodopsin (Fig. 2). However, with the addition of increasing concentrations of G$\beta\gamma$ subunits, GRK2 phosphorylation of rhodopsin is greatly increased and is further enhanced with the addition of PKA. As PKA does not significantly phosphorylate rhodopsin nor is its activity regulated by G$\beta\gamma$ subunits, the observed increase in phosphorylation is attributed to enhanced GRK2 activity toward rhodopsin.

It has been reported that PKC can directly phosphorylate GRK2 at a site within the carboxyl terminus and that this facilitates GRK2 translocation to the plasma membrane (49, 50). To investigate whether a similar mechanism exists for the phosphorylation of GRK2 by PKA, we tested the ability of PKA to phosphorylate purified full-length GRK2 and fragments of GRK2 (Fig. 3*A*). In the presence of phospholipids that activate GRK2, bovine GRK2 purified from Sf9 cells shows weak autophosphorylation (a stoichiometry of 0.03 mol of phosphate/mol of protein, Fig. 3*B*). Incubation of GRK2 with PKA leads to a significant increase in phosphorylation (0.25 mol of phosphate/mol of protein), which is further enhanced by the addition of 0.8 μM G$\beta\gamma$ subunits to 0.7 mol of phosphate/mol of protein. A fragment of the carboxyl terminus of GRK2 (residues 467–689, GRK2ct), which encompasses the pleckstrin homology domain and G$\beta\gamma$ binding domain, also acts as a good PKA substrate (0.4 mol of phosphate/mol of protein), and this phosphorylation is markedly enhanced by the addition of G$\beta\gamma$ subunits (0.7 mol of phosphate/mol of protein, Fig. 3*B*). Furthermore, a GRK2 mutant that lacks 19 residues at the carboxyl terminus is unable to be phosphorylated by PKA (Fig. 3*B*).

Examination of the extreme COOH-terminal sequence of GRK2 reveals three potential sites of PKA phosphorylation, serines 670, 676, and 685. Although the phosphorylation of serine 670 by Erk1/Erk2 was reported previously to inactivate GRK2 (44), no studies have investigated whether phosphorylation of serines 676 and 685 can affect GRK2 activity. The site of PKA phosphorylation on GRK2 was mapped by incubating the GRK2 carboxyl terminus fragment with PKA and [γ-^{32}P]ATP followed by tryptic digestion and subsequent high pressure liquid chromatography fractionation and sequencing

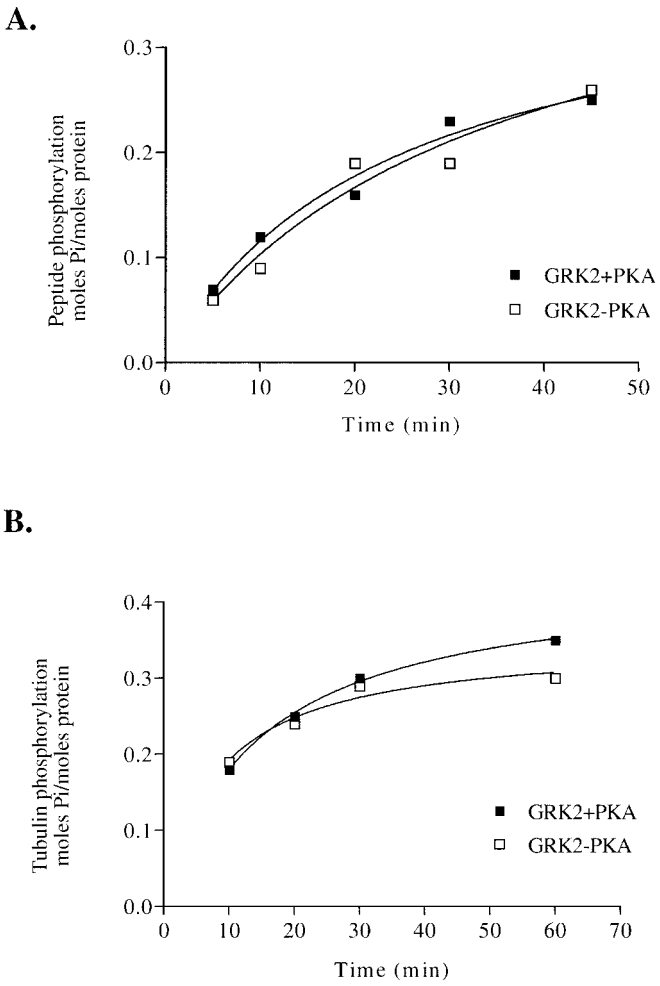

FIG. 4. **Effect of PKA on GRK2-mediated phosphorylation of peptide and tubulin.** GRK2 was incubated with [γ^{32}P]ATP and 1 mM peptide RRRREEEEESAAA (*A*) or 0.2 μg of tubulin (*B*) in the presence (□) or absence (■) of PKA. Phosphorylation reactions were performed at 30 °C for the indicated times. Incorporation of ^{32}P was measured as described under "Experimental Procedures." Basal PKA-mediated phosphorylation of tubulin has been subtracted in *lower panel*. The results are representative of three experiments.

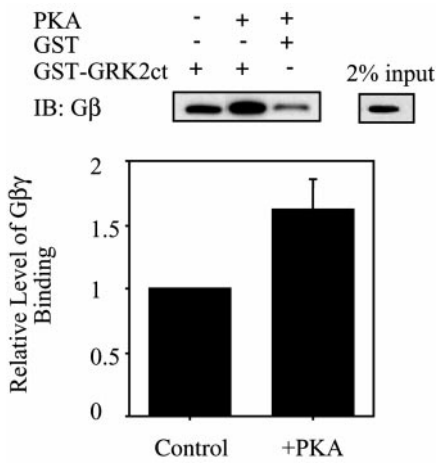

FIG. 5. **Measurement of the effect of phosphorylation of GRK2ct by PKA on G$\beta\gamma$ subunit binding.** The GST fusion protein of GRK2ct immobilized on glutathione-Sepharose beads was incubated with 4 μg of purified G$\beta\gamma$ subunits before and after phosphorylation of the fusion protein with PKA. After washing, samples were resolved by SDS-PAGE and transferred to nitrocellulose filters. The amount of G$\beta\gamma$ bound to the fusion protein or control GST was detected by immunoblotting with an anti-Gβ subunit antibody (*upper panel*). Relative amounts of G$\beta\gamma$ bound by the fusion protein with (+PKA) and without (control) phosphorylation by PKA were quantified from three experiments (*lower panel*).

of radiolabeled peptides. This yields one major phosphopeptide corresponding to residues 678–689 of GRK2 containing a single site of phosphorylation at serine 685. Taken together, these data show that PKA is capable of phosphorylating GRK2 at a specific site within the carboxyl terminus and that this phosphorylation is markedly enhanced by the presence of G$\beta\gamma$.

PKA phosphorylation of GRK2 could lead to enhanced phosphorylation of receptors by two mechanisms; either the catalytic activity of GRK2 is enhanced by its phosphorylation or GRK2 targeting to the plasma membrane is increased, possibly in a G$\beta\gamma$ subunit-dependent manner. To test whether PKA phosphorylation of GRK2 leads to a direct enhancement of GRK2 catalytic activity, the ability of GRK2 to phosphorylate nonreceptor substrates (a peptide substrate and tubulin) was compared with and without phosphorylation of GRK2 by PKA (Fig. 4). Incubation with PKA alone weakly phosphorylates tubulin and fails to phosphorylate the peptide substrate (data not shown), whereas incubation with GRK2 leads to significant phosphorylation of both substrates (0.25 mol of phosphate/mol of protein for the peptide substrate (Fig. 4*A*) and 0.3 mol of phosphate/mol of protein for tubulin (Fig. 4*B*)). Incubation of the substrates with both PKA and GRK2 under conditions that permit GRK2 phosphorylation by PKA shows no increase in the level of substrate phosphorylation, indicating that PKA phosphorylation of GRK2 does not directly affect its catalytic activity.

To test whether PKA phosphorylation of GRK2 enhances the interaction between GRK2 and G$\beta\gamma$ subunits, a GST fusion of the carboxyl terminus of GRK2 immobilized on glutathione-Sepharose beads was phosphorylated by PKA and then incubated with purified G$\beta\gamma$ subunits. After washing, the amount of G$\beta\gamma$ bound to the fusion protein was monitored by Western blotting for Gβ subunits (Fig. 5). Before phosphorylation by PKA, the GST-GRK2ct is capable of binding G$\beta\gamma$ subunits. After phosphorylation, the binding of G$\beta\gamma$ is increased by 64 ± 25%, whereas GST alone binds G$\beta\gamma$ very poorly. This finding shows that phosphorylation by PKA indeed enhances the ability of GRK2 to bind G$\beta\gamma$ subunits, and this could represent the mechanism by which GRK2 phosphorylation of receptors is regulated by factors affecting PKA activity in cells.

Upon agonist stimulation, GRK2 translocates to the plasma membrane by virtue of its interaction with G$\beta\gamma$ subunits (13–16, 51, 52). If the stability of this interaction is partly dependent on PKA-mediated phosphorylation of GRK2, the removal of PKA from the membrane should also inhibit GRK2 translocation. To test this theory, the ability of GRK2 to interact with the β2AR in cells coexpressing AKAP79 was examined (Fig. 6). Cells coexpressing the FLAG epitope-tagged β2AR, GRK2, and either AKAP79 or AKAP79$^{108-427}$ were stimulated for 5 min with 10 μM isoproterenol. Cells were then treated with a cell permeant cross-linker, and the β2AR was immunoprecipitated from the cell lysates. The amount of GRK2 present in the immune complex was detected by Western blotting. Overexpression of AKAP79$^{108-427}$ impairs GRK2 translocation to β2AR by 40 ± 15% (Fig. 6*A*), demonstrating that a mutant AKAP79 that reduces PKA association with the plasma membrane does indeed decrease GRK2 association with the β2AR.

The role that PKA phosphorylation of GRK2 plays in the colocalization of GRK2 with the β2AR was further demonstrated by mutating serine 685 in GRK2 to alanine (GRK2S685A) and comparing it to the wild type kinase in its ability to associate with the receptor. The GRK2S685A mutant lacking the PKA phosphorylation site of the native GRK2 should not show PKA-mediated enhancement of translocation.

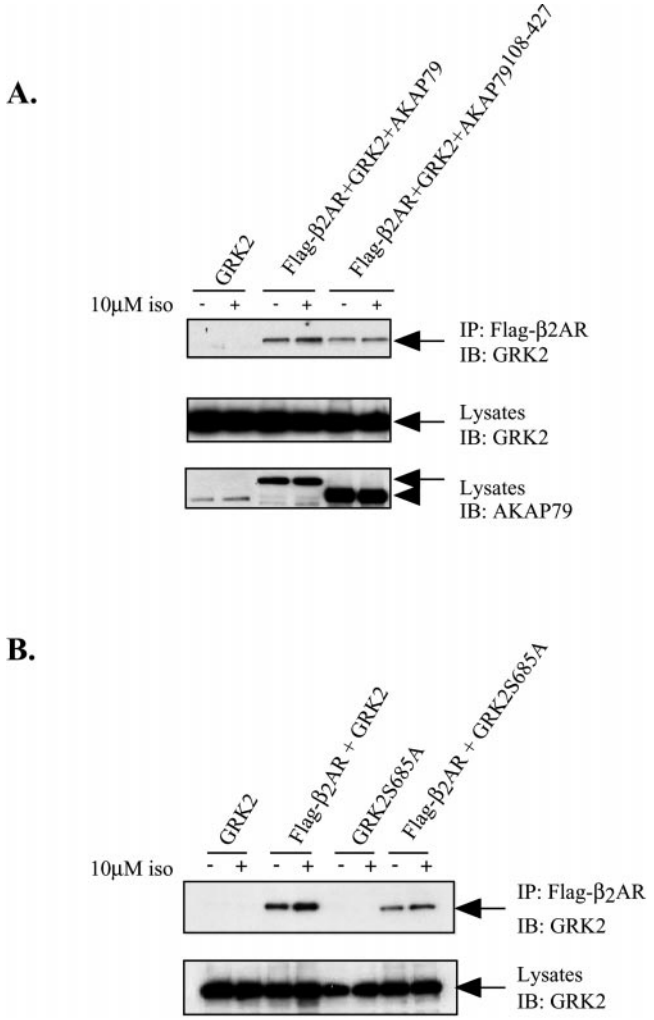

FIG. 6. **The effects of overexpression of AKAP79 and GRK2S685A on GRK2 recruitment to the stimulated β2AR.** *A,* HEK293 cells were transfected with GRK2 alone or with GRK2, the FLAG epitope-tagged β2AR, and either the wild type AKAP79 or the AKAP79$^{108-427}$ mutant. After stimulation with 10 μM isoproterenol (*iso*), the cells were treated with a cell permeant cross-linker, lysates were prepared, and the β2AR was immunoprecipitated with M2 antibody (*IP: FLAG-β2AR*). The amount of GRK2 present in the immune complex was detected by immunoblotting (*IB: GRK2*) with an anti-GRK2 monoclonal antibody (*upper panel arrow*). Expression in lysates of GRK2 (*middle panel arrow*), AKAP79 (*lower panel arrow*), and AKAP79$^{108-427}$ (*lower panel arrowhead*) were confirmed by immunoblotting. *B,* HEK293 cells were transfected with GRK2 or the GRK2S685A mutant with or without the FLAG epitope-tagged β2AR. After stimulation with 10 μM isoproterenol and cross-linking, the β2AR was immunoprecipitated (*IP: FLAG-β2AR*) with M2 antibody. The amount of GRK2 or GRK2S685A present in the immune complex (*upper panel arrow*) and in the lysate (*lower panel arrow*) was detected by immunoblotting (*IB: GRK2*). Gels are representative of three experiments.

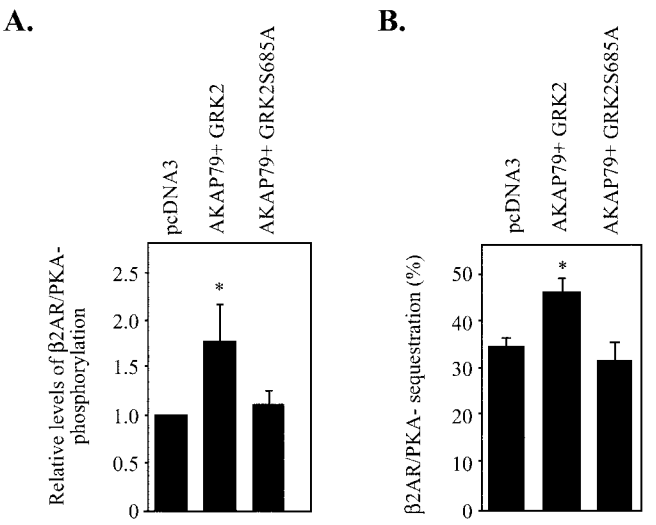

FIG. 7. **Mutation of serine 685 to alanine on GRK2 impairs GRK2-mediated β2AR/PKA− phosphorylation and internalization.** *A,* HEK293 cells expressing FLAG-β2AR/PKA− were transiently transfected with wild type GRK2 or the mutant GRK2S685A and with AKAP79. Cells were labeled with [^{32}P]orthophosphate, stimulated with 10 μM isoproterenol for 5 min, and the β2AR/PKA− was immunoprecipitated. The *graph* shows the mean of agonist-stimulated phosphorylation of β2AR/PKA− relative to nontransfected cells. The values are normalized to receptor expression levels as described under "Experimental Procedures." *B,* COS7 cells transiently transfected with FLAG-β2AR/PKA− alone or with AKAP79 and GRK2 or GRK2S685A were treated with 10 μM isoproterenol for 30 min, and the amount of receptors internalized was measured. The *graph* shows the mean ± S.E. from four experiments. *, $p < 0.05$ when compared with mock transfected cells.

Indeed, a 40% impairment in the agonist-stimulated association of GRK2S685A with β2AR compared with wild type GRK2 is observed (Fig. 6*B*).

We also predicted that coexpression AKAP79 with GRK2S685A, unlike with wild type GRK2, should not enhance β2AR/PKA− phosphorylation. When tested *in vitro* against nonreceptor substrates, the mutant and wild type GRK2 showed no detectable differences in their catalytic activities (data not shown). Coexpression of wild type GRK2 with AKAP79 enhances receptor phosphorylation and subsequently leads to enhanced levels of receptor internalization when compared with control transfected cells (Fig. 7*A*). Coexpression of GRK2S685A with AKAP79, however, fails to significantly enhance β2AR/PKA− phosphorylation, confirming that prevention of PKA-mediated phosphorylation of GRK2 at serine 685 impairs the ability of the kinase to both associate with and phosphorylate the β2AR in response to agonist. As a consequence of enhancing agonist-induced β2AR/PKA− phosphorylation by the coexpression of GRK2 and AKAP79, receptor internalization is increased (Fig. 7*B*). Coexpression of GRK2S685A and AKAP79, however, does not increase receptor internalization, mirroring the failure of the mutant kinase to enhance receptor phosphorylation.

DISCUSSION

The β2AR exists in a complex with AKAP79/AKAP150 and associated signaling proteins including PKA in rat brain tissue (27). Enhancement or perturbation of the complex in HEK293 cells leads to alterations in phosphorylation of the β2AR. This originally was assumed to occur primarily through the effects of PKA on the receptor. However, we now show that receptor phosphorylation by GRK2 is also affected by AKAP79. In HEK293 cells, the relative contributions of PKA and GRK2 to agonist-induced β2AR phosphorylation are ~20 and 80%, respectively (48). When AKAP79 is overexpressed in cells, a 40% enhancement in the level of receptor phosphorylation is observed, whereas the disruption of this complex with a mutant of AKAP79 reduces receptor phosphorylation by 50% (Fig. 1*A*). If the ability of AKAP79 to affect β2AR phosphorylation relates entirely to the phosphorylation of the receptor by PKA, then at most a 20% reduction in phosphorylation would be expected. This disparate finding led us to explore the possibility that AKAP79 somehow regulates the ability of GRKs to phosphorylate their receptor substrates. We confirmed this observation by demonstrating that phosphorylation of a mutant β2AR that is not a PKA substrate is also regulated by AKAP79 (Fig. 1, *B–D*). Furthermore, *in vitro* phosphorylation of rhodopsin by

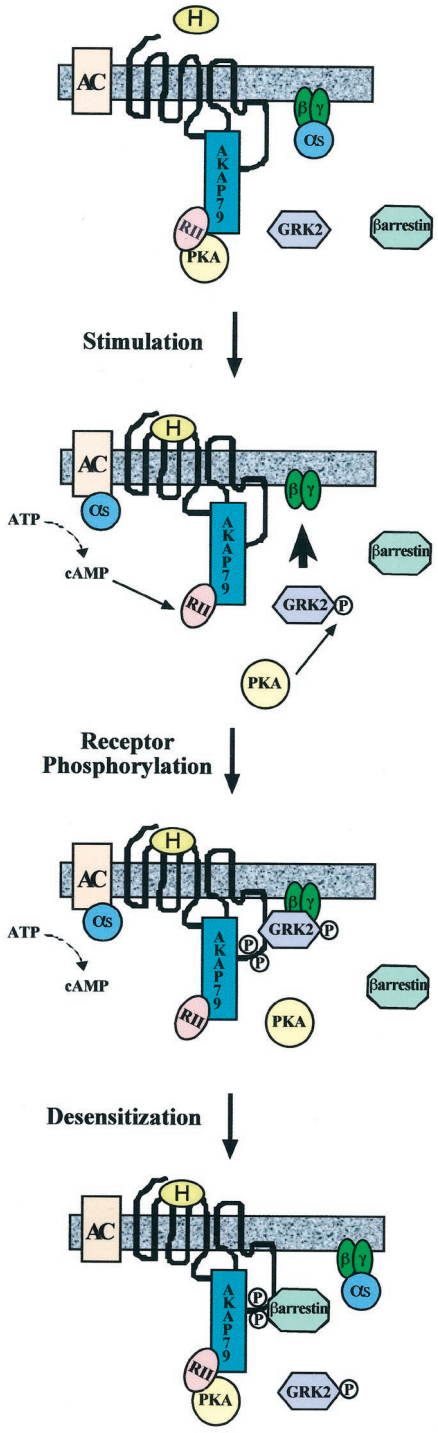

FIG. 8. ***Schematic* representation of the role of AKAP79 in regulating receptor phosphorylation by GRK2.** The receptor (*black*) in the absence of the bound hormone (*H*) is associated with PKA via its interaction with the PKA regulatory subunits II through AKAP79. On agonist binding (*Stimulation*), the receptor couples to the heterotrimeric Gs protein, leading to the dissociation of the α-subunits and $\beta\gamma$-subunits. Gαs activates the adenylate cyclase enzyme (*AC*) in the membrane, which converts ATP to cyclic AMP (*cAMP*). The binding of cAMP to the PKA regulatory subunits II causes the release of the active catalytic domain. PKA then phosphorylates GRK2 (*circled P*), enhancing its ability to interact with G$\beta\gamma$ subunits, thus promoting its translocation to the membrane. Once at the membrane, the GRK2-G$\beta\gamma$ complex stabilized by the phosphorylation of GRK2 by PKA can associate better with and phosphorylate the agonist-occupied receptor. After phosphorylation, the β-arrestin proteins bind to the receptor leading to desensitization and attenuation of G protein coupling.

GRK2 is enhanced in the presence of PKA and G$\beta\gamma$ subunits, even though rhodopsin itself is not a PKA substrate (Fig. 2).

The mechanism underlying these findings appears to be the ability of PKA to phosphorylate GRK2 at a specific residue, serine 685 (Fig. 3). Phosphorylation at this site does not alter the kinase activity of GRK2 *per se* (Fig. 4). Rather, it leads to the enhanced binding of GRK2 to G$\beta\gamma$ subunits, which should increase the association of the kinase with receptor substrates (Fig. 5). We also demonstrate that the ability of PKA to phosphorylate GRK2 *in vitro* is enhanced in the presence of G$\beta\gamma$ subunits, suggesting that in cells PKA may preferentially phosphorylate those GRK2 molecules already complexed with G$\beta\gamma$ subunits at the membrane. Therefore, phosphorylation by PKA may be involved both in promoting translocation of GRK2 to the membrane and in maintaining GRK2 in a G$\beta\gamma$-bound form once there.

We confirmed the role of PKA phosphorylation in GRK2 membrane translocation by showing that the mutation of the PKA phosphorylation site in GRK2 (serine 685 to alanine) diminishes the ability of GRK2 to be recruited to β2AR and phosphorylate the β2AR (Figs. 6 and 7). The phosphorylation of GRK2 by PKA was also shown to have a functional consequence as overexpression of GRK2, but not GRK2S685A, leads to enhanced receptor internalization in response to agonist (Fig. 7*B*). Tethering of PKA to the β2AR via AKAP79, therefore, may represent a regulatory loop whereby agonist-stimulated coupling of the β2AR to Gs, resulting in the production of cAMP, is attenuated by the cAMP-activated kinase PKA phosphorylating both the receptor and the major desensitizing kinase, GRK2. This process leads to increased translocation of GRK2 to the receptor by enhanced binding to G$\beta\gamma$ subunits, resulting in further receptor phosphorylation, desensitization, and internalization (Fig. 8).

A similar mechanism may also exist for the recruitment of GRK2 to the receptor following phosphorylation of GRK2 by PKC. Earlier studies have shown that activation of the cellular pool of PKC with phorbol 12-myristate 13-acetate leads to the translocation of cytosolic GRK2 to the plasma membrane (49, 50). A site of robust PKC phosphorylation on GRK2 has been mapped partially to within the COOH-terminal domain, and it has been demonstrated that such phosphorylation leads to enhanced G$\beta\gamma$-dependent GRK2 activity toward a purified receptor substrate.

AKAP79 acts as a scaffolding protein for both PKA and PKC, associating both kinases with the plasma membrane and the β2AR. Therefore, it is feasible that AKAP79 also modulates the ability of PKC to affect membrane recruitment of GRK2. If this is the case, AKAP79 might represent a general scaffold for regulating membrane recruitment of GRK2 by second messenger-activated kinase phosphorylation. It will be interesting to determine whether the site of PKC phosphorylation on GRK2 is the same as or different from that of PKA and whether such phosphorylation also enhances G$\beta\gamma$ binding. It is also worthy to note that GRK5 can be phosphorylated by PKC at sites near the carboxyl terminus, but unlike GRK2, this phosphorylation inhibits GRK5 activity (53). Similarly, phosphorylation of GRK2 by Erk1 or Erk2 on serine 670 leads to an almost complete inhibition of the kinase (44). This may represent a mechanism by which GRK2 is returned to an inactive state after it has phosphorylated agonist-occupied receptors. PKC phosphorylation of GRK5, therefore, seems more analogous to the Erk1/Erk2 phosphorylation of GRK2 rather than to GRK2 phosphorylation by either PKA or PKC. These marked differences in regulatory phosphorylation imply that the roles of these two GRKs in GPCR regulation are very different.

It has been reported recently that gravin (AKAP250) can also

form complexes with the β2AR receptor and that it can affect both receptor desensitization and resensitization (30, 54). It is not yet clear what the exact role of gravin is in these processes, but it is likely to be dependent on the ability of gravin to associate with both second messenger-activated kinases and specific protein phosphatases. The ability of the β2AR to associate with more than one AKAP protein, each with distinct yet overlapping sets of associated signaling molecules, leads to the intriguing possibility that different steps during receptor regulation are modulated by different AKAPs. These steps may require the physical separation of signaling components onto distinct scaffolds, allowing entire complexes to be recruited to the receptor at specific stages during desensitization and resensitization. AKAP79 is constitutively associated with the β2AR, and thus it may be required at many stages during receptor regulation (27) including early events such as the recruitment of GRK2. The association of the receptor with gravin, however, is regulated by agonist and therefore may be required for more specific events in the desensitization/resensitization process.

Our studies implicate a role for AKAP79 and PKA in the regulation of GRK2 recruitment to agonist-occupied β2AR molecules in a manner that is mediated by the enhanced binding of GRK2 to Gβγ subunits. This function of a scaffold protein and PKA in the regulation of GRK2 activity represents a novel mechanism by which two kinases activated during β2AR signaling can influence receptor desensitization.

Acknowledgments—We thank Drs. Randy A. Hall, Richard Premont, Audrey Claing for helpful discussion, Millie McAdams and Judy Phelps for DNA sequencing, and Donna Addison and Mary Holben for assistance with the preparation of this manuscript.

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