G Protein-coupled Receptor Kinase 5 Regulates β₁-Adrenergic Receptor Association with PSD-95*

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We previously reported that the β₁-adrenergic receptor (β₁AR) associates with PSD-95 through a PDZ domain-mediated interaction, by which PSD-95 modulates β₁AR function and facilitates the physical association of β₁AR with other synaptic proteins such as N-methyl-D-aspartate receptors. Here we demonstrate that β₁AR association with PSD-95 is regulated by G protein-coupled receptor kinase 5 (GRK5). When β₁AR and PSD-95 were coexpressed with either GRK2 or GRK5 in COS-7 cells, GRK5 alone dramatically decreased the association of β₁AR with PSD-95, although GRK2 and GRK5 both could be co-immunoprecipitated with β₁AR and both could enhance receptor phosphorylation in vivo. Increasing expression of GRK5 in the cells led to further decreased β₁AR association with PSD-95. Stimulation with the β₁AR agonist isoproterenol further decreased PSD-95 binding to β₁AR. In addition, GRK5 protein kinase activity was required for this regulatory effect since a kinase-inactive GRK5 mutant had no effect on PSD-95 binding to β₁AR. Moreover, the regulatory effect of GRK5 on β₁AR association with PSD-95 was observed only when GRK5 was expressed together with the receptor, but not when GRK5 was coexpressed with PSD-95. Thus, we propose that GRK5 regulates β₁AR association with PSD-95 through phosphorylation of β₁AR. Regulation of protein association through receptor phosphorylation may be a general mechanism used by G protein-coupled receptors that associate via PDZ domain-mediated protein/protein interactions.

PDZ domains, named after the first three proteins in which they were discovered (PSD-95/Dlg/ZO-1), bind to small specific primary sequences in their target proteins, typically a carboxyl-terminal peptide (1). PDZ domain-mediated protein/protein interactions have been identified that target many receptors, ion channels, enzymes, and other structural molecules (2, 3). PDZ domain-containing scaffolding proteins have been shown to play very important roles in organizing signaling cascades in a specific spatial order, anchoring proteins in specific subcellular compartments, and regulating ion channel activity (2–6).

A variety of G protein-coupled receptors (GPCRs)$^3$(including the β₁-adrenergic receptor (β₁AR), β₁AR, vasoactive intestinal peptide, V2 vasopressin, metabotropic glutamate, serotonin, and somatostatin receptors) contain typical class I PDZ domain-interacting motifs of the form (S/T)(V/I/L) at their carboxyl termini (7). β₁AR was the first GPCR reported to interact with a PDZ domain-containing protein (8). Agonist-stimulated binding of the first PDZ domain of the Na⁺/H⁺ exchanger regulatory factor (NHERF) to the extreme C terminus of β₁AR provides a mechanism by which β₁AR controls Na⁺/H⁺ exchanger NHE3 function in the kidney (8). Furthermore, interaction with NHERF has been reported to regulate the recycling of internalized β₁AR (9). Recently, additional PDZ domain-containing proteins interacting with several distinctGPCRs have been identified. MUPP1, a multi-PDZ domain-containing protein, has been demonstrated to interact with three subtypes of serotonin receptors through its PDZ10 domain (10). STTRIP has been identified as a novel binding partner for somatostatin receptor subtype 2 (11, 12). Cortactin-binding protein 1 also has been reported to interact with somatostatin receptor subtype 2 in an agonist-dependent manner (13). Another PDZ domain-containing protein, PICK1 (protein interacting with C kinase), has been reported to interact with protein kinase C (PKC) and to regulate its phosphorylation of G protein-coupled metabotropic glutamate receptor subtype 7a (14).

Using yeast two-hybrid screening, we recently identified PSD-95, a postsynaptic density-enriched protein, as a novel binding partner of β₁AR (15). PSD-95 belongs to the large family of membrane-associated guanylate kinase domain proteins that contain multiple PDZ domains. PSD-95 is known to interact with several proteins, including N-methyl-D-aspartate receptors (16), K⁺ channels (1), the receptor-tyrosine kinase ErbB4 (17), and nitric-oxide synthase (18, 19), through its three PDZ domains. PSD-95 is thought to play critical roles in assembling these and other molecules into signaling complexes and regulating their signal transduction (20). Association with PSD-95 decreases agonist-induced β₁AR internalization, but has no effect on receptor desensitization or receptor-induced cAMP accumulation (15). This association provides a physical linkage between β₁ARs and other synaptic proteins, such as N-methyl-D-aspartate receptors, which are known to be regulated by β₁AR stimulation (21). Association with PSD-95 may also provide a molecular mechanism by which β₁AR may be localized to synapses and regulate synaptic plasticity.

If PDZ domain-containing proteins that interact with the carboxyl terminus of a GPCR via a PDZ domain do indeed serve as multivalent scaffolding proteins to selectively assemble and target receptor-containing signaling complexes to specific cellular areas (10, 12, 15), this assembly is likely to be highly regulated. The assembly of receptor signaling complexes on the

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$^3$ The abbreviations used are: GPCRs, G protein-coupled receptors; β₁AR, β₁-adrenergic receptor; β₁AR, β₁-adrenergic receptor; NHERF, Na⁺/H⁺ exchanger regulatory factor; PKC, protein kinase C; GRK, G protein-coupled receptor kinase; GST, glutathione S-transferase; AMPA, α-amino-3-hydroxy-5-methylisoxazole propionate.
cell surface is a dynamic process, and the complexes must be able to disassemble under certain physiological conditions. Despite the fact that several protein/protein interactions between GPCRs and PDZ domain-containing proteins have been well documented, little is known about how such PDZ domain-mediated interactions might be regulated.

G protein-coupled receptor kinases (GRKs) constitute a family of seven serine/threonine-protein kinases that phosphorylate agonist-bound, activated GPCRs. GRK-mediated receptor phosphorylation rapidly initiates receptor desensitization and internalization through clathrin-mediated endocytosis by targeting the receptors for binding by arrestin proteins (22, 23). It has been reported that β2AR, the serine/threonine-rich cytoplasmic tail is the target region phosphorylated by GRKs (24). In vitro experiments have indicated that GRK2 and GRK5 preferentially phosphorylate some of these sites (24). Both β2AR and β1AR have carboxyl-terminal PDZ target motifs that have been reported to bind to PDZ domain-containing proteins (8, 15). NHERF binds to the DSSL-COOH motif of β2AR, and PDZ-95 binds to the ESKV-COOH motif of β4AR. In both cases, mutation of the serine at position −2 eliminates PDZ domain-containing protein binding. Interestingly, this Ser residue at position −2 in β2AR has been mapped as a phosphorylation site for GRK5, but not for GRK2 (24). Even though nothing is known about the GRK phosphorylation sites in β3AR, it has been reported that β3AR can be phosphorylated and desensitized by both GRK2 and GRK5 in a manner similar to that for β2AR (25). Considering that the protein sequences in β2AR and β1AR are highly conserved in the carboxyl-terminal tail, it is reasonable to believe that the Ser residue in the ESKV-COOH motif of β2AR may also be a phosphorylation site for GRKs, and phosphorylation of this site may affect receptor interaction with its binding partner. Therefore, we investigated whether GRKs might regulate β2AR interaction with PDZ-95.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Mammalian expression plasmids pcDNA3/FLAG-β2AR and pcDNA3/FLAG-β1AR (26); pCMV5/His-β-arrestin-2 (27); and pRK5/GRK2, pRK5/GRK5, and pRK5/GRK5(K215R) (GRK5(DN), where DN is dominant-negative) (25, 28) have been described previously. Plasmid GW1/Myc-PSD-95 (1) was a generous gift from Dr. Morgan Sheng (Harvard Medical School). Polyclonal anti-His antibody and polyclonal (A14) and monoclonal (9E10) anti-Myc antibodies were from Santa Cruz Biotechnology. Anti-FLAG M2 and anti-FLAG BioM2 antibodies, fluorescein isothiocyanate-conjugated anti-mouse IgG, and anti-FLAG M2 affinity gel were from Sigma. Monoclonal anti-GRK2 and anti-GRK5 antibodies were developed in this laboratory as described previously (29). Horseradish peroxidase-conjugated streptavidin and anti-mouse IgG and anti-rabbit IgG secondary antibodies were from Amersham Biosciences, Inc.

Cell Culture and Transfection—All tissue culture media and related reagents were purchased from In Vitrogen. COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin in a 37 °C incubator under 5% CO2. Cells in 100-mm dishes were transfected with LipofectAMINE 2000 (at a 2.5:1 ratio with DNA; Roche Molecular Biochemicals) according to the manufacturer’s protocol. HEK293 cells were transfected with FuGene 6 (at a 4:1 ratio with DNA) according to the manufacturer’s protocol. After transfection, cells were washed with cold Dulbecco’s phosphate-buffered saline and replaced with 1 ml ice-cold lysis buffer (Dulbecco’s phosphate-buffered saline containing 10 mM HEPES, pH 7.4, and a 2.5 mM concentration of the cell-permeable cross-linking reagent dithiobis(succinimidyl propionate) (Pierce)) (30). For the stimulated cells, 10 μM isoproterenol was present in the cross-linking buffer. The cells were incubated for 30 min at room temperature with continuous slow rocking. The cross-linking reaction was terminated by quickly removing the cross-linking buffer and replacing it with 1 μl of ice-cold lysis buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors). The cell lysates were clarified as described above, and the supernatants were used for immunoprecipitation experiments.

Clariﬁed cell extract (1 ml) was then incubated with 25 μl of anti-FLAG M2 affinity gel slurry at 4 °C with gentle rotation for 4 h. Beads were washed four to five times with ice-cold lysis buffer B. Lysates and bound proteins were eluted with 50 μl of 3× SDS-PAGE sample buffer. Equivalent amounts of sample in 3× SDS-PAGE sample buffer were resolved on pre-cast 4–20% polyacrylamide gel (Invitrogen) and then transferred to nitrocellulose membrane by semidry blotting. Nitrocellulose membranes were blocked with 5% fat-free milk in TBST (20 mM Tris, pH 7.4, 500 mM NaCl, and 0.1% Tween 20) and incubated with the appropriate primary antibody at room temperature for 1 h. PSD-95 was detected with rabbit anti-Myc antibody (1:1000 dilution); β-arrestin-1 and β-arrestin-2 were detected with rabbit anti-His antibody; GRK2 was detected with GRK2/3-specific monoclonal antibody C5/7; and GRK5 was detected with GRK4/5/6-specific monoclonal antibody A16/17. FLAG-tagged receptor proteins in the immunoprecipitation were blotted with BioM2 IgG. After washing with TBST, membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1:2000 dilution) or with horseradish peroxidase-conjugated streptavidin (for BioM2). Protein bands were visualized via SuperSignal chemiluminescence substrate (Pierce). Quantitation of band density was performed with a Bio-Rad Fluor-S Multimeter.

In Vitro Glutathione S-Transferase (GST) Fusion Protein Phosphorylation—COS-7 cells in 100-mm dishes were transiently transfected with FLAG-β2AR alone or with either GRK2 or GRK5. 48 h after transfection, cells were washed with phosphate-free Dulbecco’s modified Eagle’s medium and incubated with [32P]orthophosphate to label the intracellular ATP pool as described previously (31). Cells were then stimulated with 10 μM isoproterenol for 5 min and washed twice with ice-cold Dulbecco’s phosphate-buffered saline. Cells were solubilized in 3× SDS-PAGE sample buffer and subjected to immunoprecipitation with anti-FLAG M2 affinity gel. Immunoprecipitates were resolved on 10% polyacrylamide gels. The gels were stained with Coomassie Blue, dried, and subjected to autoradiography. Receptor phosphorylation was quantitatively analyzed with a Molecular Dynamics PhosphorImager as described previously (25).

Receptor Internalization—For receptor internalization assays, HEK293 cells in 100-mm dishes were transiently transfected with pcDNA3/FLAG-β2AR or pcDNA3/FLAG-β1AR in the absence and presence of GW1/Myc-PSD-95. One day after transfection, cells were split into polylysine-coated six-well plates (Biosco) and grown overnight at 5 µg/ml leupeptin, 20 µg/ml phenylmethylsulfonfluoride, 10 mM NaF, and 1 mM sodium orthovanadate). The cells were lysed by incubation on ice for 30 min and then clarified by centrifugation at 21,000 g for 12 min at 4 °C. The clarified supernatants were used in cellular co-immunoprecipitation experiments. 50 μl of each supernatant were diluted into an equal amount of 3× SDS-PAGE sample buffer and served as a whole cell extract control.

For protein cross-linking experiments, transfected COS-7 cells in 100-mm plates were incubated in serum-free medium for 60 min and then exposed to 10 μM isoproterenol for 10 min. The cells were rinsed with cold Dulbecco’s phosphate-buffered saline and replaced with 1 ml ice-cold lysis buffer (Dulbecco’s phosphate-buffered saline containing 10 mM HEPES, pH 7.4, and 2.5 mM concentration of the cell-permeable cross-linking reagent dithiobis(succinimidyl propionate) (Pierce)) (30). For the stimulated cells, 10 μM isoproterenol was present in the cross-linking buffer. The cells were incubated for 30 min at room temperature with continuous slow rocking. The cross-linking reaction was terminated by quickly removing the cross-linking buffer and replacing it with 1 μl of ice-cold lysis buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors). The cell lysates were clarified as described above, and the supernatants were used for immunoprecipitation experiments. Proteins were eluted with 50 μl of 3× SDS-PAGE sample buffer. Equivalent amounts of sample in 3× SDS-PAGE sample buffer were resolved on pre-cast 4–20% polyacrylamide gel (Invitrogen) and then transferred to nitrocellulose membrane by semidry blotting. Nitrocellulose membranes were blocked with 5% fat-free milk in TBST (20 mM Tris, pH 7.4, 500 mM NaCl, and 0.1% Tween 20) and incubated with the appropriate primary antibody at room temperature for 1 h. PSD-95 was detected with rabbit anti-Myc antibody (1:1000 dilution); β-arrestin-1 and β-arrestin-2 were detected with rabbit anti-His antibody; GRK2 was detected with GRK2/3-specific monoclonal antibody C5/7; and GRK5 was detected with GRK4/5/6-specific monoclonal antibody A16/17. FLAG-tagged receptor proteins in the immunoprecipitation were blotted with BioM2 IgG. After washing with TBST, membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1:2000 dilution) or with horseradish peroxidase-conjugated streptavidin (for BioM2). Protein bands were visualized via SuperSignal chemiluminescence substrate (Pierce). Quantitation of band density was performed with a Bio-Rad Fluor-S Multimeter.

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RESULTS

Expression of GRK5 Decreases PSD-95 Binding to $\beta_1$AR—To test the effect of GRKs on the association of $\beta_1$AR with PSD-95, we transfected COS-7 cells with FLAG-$\beta_1$AR and Myc-PSD-95 in the absence or presence of either GRK2 or GRK5. As reported previously (15), in the absence of exogenous GRKs, immunoprecipitation of $\beta_1$AR followed by Western blotting revealed robust co-immunoprecipitation of a PSD-95-$\beta_1$AR complex (Fig. 1A). Treatment with the $\beta$-adrenergic receptor agonist isoproterenol had no significant effect on this association in the absence of GRKs. However, expression of GRK5 dramatically decreased the association of PSD-95 with $\beta_1$AR, whereas the association of $\beta_1$AR with PSD-95 was not altered by coexpression of GRK2 (Fig. 1A). With an increased expression level, GRK5 further decreased the amount of PSD-95 binding to the receptor (Fig. 1B), whereas an increased expression level of GRK2 was ineffective (Fig. 1C).

Furthermore, the negative effect of GRK5 on PSD-95/$\beta_1$AR binding was potentiated by agonist stimulation of $\beta_1$AR (Figs. 1A and 2). In the cells lacking GRK5, 10 min of stimulation with isoproterenol did not change the association of $\beta_1$AR with PSD-95. However, expression of even a small amount of GRK5 dramatically decreased the association of PSD-95 with $\beta_1$AR (Figs. 1B and 2). In the presence of GRK5, isoproterenol stimulation further decreased the amount of PSD-95 associated with the receptor (Figs. 1A and 2). With extended stimulation by agonist, the amount of PSD-95 bound to $\beta_1$AR was further decreased (Fig. 2). This agonist effect could result from 1) agonist stimulation activating the receptor and changing its conformation, thus presenting a better substrate for GRK5, or 2) activated GPCR enhancing GRK5 kinase activity (23). In both cases, agonist stimulation led to enhanced receptor phosphorylation by GRK5. These results suggest that receptor phosphorylation might be responsible for the GRK5 effect on PSD-95/$\beta_1$AR binding. The reason why no agonist effect was observed in the cells in the absence of GRK5 may be because there is not a sufficient amount of endogenous GRK5 (compared with GRK2) in the COS-7 cells. In fact, Western blotting of COS-7 cell lysate suggested that there is a significantly higher level of GRK2 than of GRK5 in these cells (data not shown).

Both GRK2 and GRK5 Can Associate with $\beta_1$AR and Enhance Its Phosphorylation—It has been well documented that many GPCRs are substrates for both GRK2 and GRK5, as they can be phosphorylated by GRK2 or GRK5 both in vitro and in vivo (23). Furthermore, GRK2 and GRK5 have been demonstrated to associate with GPCRs in an agonist-dependent manner (30, 31). Here we tested the effect of GRK2 and GRK5 on $\beta_1$AR phosphorylation and their association with $\beta_1$AR in COS-7 cells. As has been previously shown (25), both GRK2 and GRK5 can enhance the phosphorylation of $\beta_1$AR. In the cells expressing $\beta_1$AR alone, agonist stimulation caused little increase in receptor phosphorylation. However, we observed a dramatic increase in agonist-stimulated receptor phosphorylation when $\beta_1$AR was coexpressed with either GRK2 or GRK5 in COS-7 cells (Fig. 3A).

The interaction between GRKs and their substrate receptors is so weak (or transient) that co-immunoprecipitation of GRK5/$\beta_1$AR is undetectable under standard immunoprecipitation conditions (data not shown). However, GRK5/$\beta_1$AR association is detectable following treatment of the stimulated cells with the membrane-permeable cross-linking reagent dithiobis(succinimidyl propionate). As shown in Fig. 3B, when GRK2 or
GRK5 was coexpressed with β1AR in COS-7 cells, immunoprecipitation of the receptor revealed robust association of GRK2 and GRK5 with the receptor (Fig. 3B).

**GRK5 Kinase Activity Is Required to Regulate the β1AR Association with PSD-95**—Since both GRK2 and GRK5 are co-immunoprecipitated with β1AR, it is possible that the GRK5 inhibition of PSD-95/β1AR association could be due to competition between PSD-95 and GRK5 to binding to the receptor. To address this question, we used a GRK5 mutant, K215R (GRK5(DN)), which has no kinase activity. When β1AR and PSD-95 were coexpressed with differing amounts of GRK5(DN), the association of PSD-95 with β1AR was not significantly decreased (Fig. 4), even though GRK5(DN) still retains the same ability to co-immunoprecipitate with the receptor as wild-type GRK5 (data not shown). These data indicate that the ability GRK5 to reduce receptor association with PSD-95 is not due to competition between GRK5 and PSD-95 for receptor binding, but requires the protein kinase activity of GRK5. This conclusion is also supported by the preceding results that agonist stimulation potentiated the ability of GRK5 to dissociate PSD-95 from β1AR (Fig. 2).

To quantitatively determine the effect of GRKs on PSD-95/β1AR association, we analyzed the PSD-95 immunoreactive protein bands using a Bio-Rad Fluor-S MultiImager. We set the amount of PSD-95 associated with β1AR in the absence of exogenous GRKs and without agonist stimulation as the basal level (100%), and all other results are expressed as a percentage of this basal level. As shown in Fig. 5, when β1AR and PSD-95 were coexpressed with either GRK2 or the GRK5(K215R) mutant (GRK5(DN)), the association of β1AR with PSD-95 was not significantly changed. Agonist stimulation of these cells also had no effect on PSD-95/β1AR association. However, coexpression of GRK5 dramatically decreased the binding of β1AR with PSD-95, down to 24% of the control level. Agonist stimulation further decreased the amount of PSD-95 bound to the receptor to 5% of the basal level (Fig. 5).

**GRK5 Regulates PSD-95/β1AR Association by Phosphorylation of the Receptor**—GRK5 could phosphorylate either β1AR or PSD-95 to regulate the association of PSD-95 with β1AR. To ascertain the phosphorylation target of GRK5, we expressed β1AR, PSD-95, β1AR with GRK5, or PSD-95 with GRK5 in COS-7 cells. Lysate from cells expressing β1AR was then mixed with that from cells expressing PSD-95, followed by immunoprecipitation of β1AR. As shown in Fig. 6, when GRK5 was coexpressed with PSD-95, it was unable to inhibit PSD-95 binding to β1AR. However, when GRK5 was coexpressed with β1AR, the association of PSD-95 with β1AR was decreased. Since the lysate contains no additional ATP, GRK5 should be catalytically inactive once the cells are lysed, suggesting that GRK5 regulates β1AR association with PSD-95 by phosphorylation of β1AR, not PSD-95.

**PSD-95/β1AR Association Is Not Affected by β-Arrestins**—Following phosphorylation of the agonist-activated receptor by GRKs, β-arrestins bind to the receptor and promote receptor internalization (22). Thus, β-arrestin binding to the receptor might prevent PSD-95/β1AR association. To investigate whether β-arrestin binding is required for regulating PSD-95/β1AR association, we tested the effect of β-arrestins on PSD-95/β1AR association in the presence of GRK5. COS-7 cells were transfected with either β-arrestin-1 or β-arrestin-2 with β1AR, PSD-95, and GRK5. As shown in Fig. 7, the amount of PSD-95 associated with β1AR was decreased by expression of GRK5. However, the association of PSD-95 with β1AR was not significantly further changed with overexpression of either β-arrestin-1 or β-arrestin-2 in the presence of GRK5. We also noted that overexpression of β-arrestins did not facilitate GRK2 reg-
FK. 4. Kinase-inactive GRK5 mutant has no effect on PSD-95 binding to β1AR. COS-7 cells were transiently transfected with β1AR and PSD-95 together with GRK2, GRK5, or various amounts of the kinase-inactive or dominant-negative GRK5(K215N) mutant (GRK5(DN)). The receptors were immunoprecipitated (IP) with anti-FLAG M2 affinity gel. PSD-95 bound to the beads was resolved by SDS-PAGE and detected with anti-Myc antibody. GRK2 and GRK5 in the cell lysates were detected with a mixture of monoclonal anti-GRK2 and anti-GRK5 antibodies. Expression of PSD-95 and GRKs in the cell lysates is shown for comparison. The data shown are representative of four independent experiments. ISO, isoproterenol.

Fig. 5. Summary of the effect of GRKs on PSD-95 binding to β1AR. In each experiment, the bands corresponding to PSD-95 were quantitated on a Bio-Rad Fluor-S MultiImager. Cells expressing only PSD-95 and β1AR were used as a control, and the amount of PSD-95 associated with the receptor in the absence of agonist stimulation in these control cells was considered as the basal level (100%). PSD-95 amounts bound to the receptors in the presence of cotransfected GRK2, GRK5, and GRK5(DN) (3 μg of DNA) were normalized to this level and are expressed as a percentage of the control. The white and black bars represent samples in the absence and presence of agonist stimulation (isoproterenol [ISO], 10 min), respectively. The data are presented as means ± S.E. from four independent experiments.

Fig. 6. GRK5 regulate PSD-95 binding to β1AR by phosphorylation of the receptor. COS-7 cells were transiently transfected with β1AR, PSD-95, β1AR with GRK5, or PSD-95 with GRK5. The cells overexpressing GRK5 were stimulated with 10 μM isoproterenol for 10 min and then solubilized as described under “Experimental Procedures.” Equal amounts of cell lysates were mixed as indicated. β1ARs were then immunoprecipitated (IP) from the mixture with anti-FLAG M2 beads. PSD-95 bound to the beads was resolved by SDS-PAGE and blotted with anti-Myc antibody. Expression of PSD-95 and GRK5 in the cell lysates was determined by probing with anti-Myc and anti-GRK5 antibodies, respectively. The data shown are representative of three independent experiments.

Fig. 7. Overexpression of β-arrestin does not affect PSD-95 binding to β1AR. COS-7 cells were transiently transfected with FLAG-β1AR, Myc-PSD-95, pRK5/GRK5, and either β-arrestin-1 (Barr 1) or β-arrestin-2 (Barr 2) as indicated. Cells were serum-starved for 60 min before being treated with 10 μM isoproterenol (ISO) for 10 min. FLAG-tagged receptors were immunoprecipitated (IP) with anti-FLAG M2 beads, and bead-bound PSD-95 was resolved by SDS-PAGE and blotted with anti-Myc antibody as described under “Experimental Procedures.” Expression of PSD-95, β-arrestins, and GRKs in the cell lysates is shown for comparison. The results shown are representative of three independent experiments.

Fig. 8. GRK2 (Fig. 8). These results suggest that, at least in vitro, β1AR-CT is a better substrate for GRK5 than for GRK2.

DISCUSSION

Phosphorylation of GPCRs by GRKs is a major mechanism responsible for termination of cellular signaling by these receptors or desensitization (33). Among the somatic GRKs, GRK2, GRK3, and GRK5 have been reported to enhance the phosphorylation of β1AR in HEK293 cells (25). Based on sequence and functional similarities, GRK2 and GRK3 belong to one GRK subfamily, whereas GRK5 belongs to a distinct subfamily that includes GRK4, GRK5, and GRK6 (23). Here we studied the ability of GRKs to regulate a PDZ domain-mediated interaction with β1AR by using GRK2 and GRK5, two enzymes from different subfamilies. We have demonstrated that phosphorylation of β1AR by GRK5, but not by GRK2, appears to regulate its binding to one intracellular protein-binding partner, PSD-95. Several recent reports suggest that phosphorylation can reg-
GRK5 Regulates PSD-95/β2AR Association

![Diagram](image)

**Fig. 9.** Protein sequence alignment of the extreme carboxyl termini of β1AR and β2AR. The arrows indicate residues in both receptors that are critical for binding with PDZ-domain-containing proteins. The serine at position -2 is the putative phosphorylation site for GRK5.

**Fig. 8.** The β2AR carboxyl terminus is a better substrate for GRK5 than for GRK2. Equal amounts of GST fusion proteins (GST, GST-β1CT, and GST-β2CT) immobilized on glutathione-Sepharose 4B beads were incubated with equal amounts of purified GRK2 or GRK5 in the presence of [γ-32P]ATP. GRK2- or GRK5-mediated GST fusion protein phosphorylation was analyzed as described under “Experimental Procedures.” The data shown are representative of three independent experiments.

GRK5 can phosphorylate agonist-bound receptors (28). This phosphorylation of the receptor by GRK5 decreases the binding affinity for glutamate receptor-interacting protein (34). Furthermore, serine phosphorylation has also been implicated in the differential regulation of the α-aminono-3-hydroxy-5-methylisoxazole propionate (AMPA) receptor interaction with PDZ domain-containing proteins (35). Phosphorylation of the carboxyl terminus of the inward rectifier K+ channel (Kir2.3) by protein kinase A inhibits its association with PSD-95 and SAP97 (36, 37). In fact, for all class 1 PDZ domain-interacting motifs, there is a phosphorylatable serine or threonine at position -2 that is critical for PDZ domain-containing protein binding. Phosphorylation of the serine or threonine at this site may provide an efficient way to regulate PDZ domain-mediated protein interactions.

β2AR interacts with the PDZ-domain-containing protein NHERF through the PDZ1 domain (8, 38). This PDZ domain-mediated interaction of β2AR with NHERF family proteins has been shown to control the choice between degradation and recycling of internalized β2AR (9). Very interestingly, the recycling of β2AR was dependent on the intact PDZ domain-binding motif (DSLL-COOH) in the carboxyl terminus of β2AR (Fig. 9), and receptor recycling is modulated by overexpression of GRK5, but not of GRK2 (9). The Ser residue in this motif has been shown to be a phosphorylation site for GRK5, but not for GRK2, in vitro (Fig. 9) (24). It is likely that phosphorylation of this Ser residue by GRK5 can decrease the binding of NHERF or other proteins to β2AR and thereby regulate the recycling of the internalized receptors. Interestingly, one splice variant of GRK6 (GRK6A) can phosphorylate NHERF at Ser289 as well, also suggesting that GRKs may regulate PDZ domain-mediated interactions (39).

Here we have shown that phosphorylation of β1AR by GRK5 decreases the association of β1AR with PSD-95. It has been reported that β1AR can be phosphorylated by GRKs in a manner analogous to that of β2AR (25). The ESKV-COOH motif in the carboxyl terminus of β1AR appears to be responsible for the interaction with PSD-95 (15). The Ser residue at position -2 (Ser475) is the only putative GRK phosphorylation site in this motif (Fig. 9). One explanation for our observation is that this Ser residue is phosphorylated by GRK5, but not by GRK2, and that phosphorylation of this Ser residue decreases the affinity of PSD-95 binding. We have shown that the β2AR carboxyl-terminal tail is a better substrate for GRK5 than for GRK2, but further studies will be required to assess whether phosphorylation of Ser475 indeed results from the action of GRK5 in vivo and whether this particular phosphorylation underlies all of the GRK5 regulatory effect on PSD-95 association. Thus, for both β1AR and β2AR, association with PDZ domain-containing partners is specifically regulated by GRK5, not GRK2. In view of the fact that many GPCRs have the similar serine/threonine-based PDZ domain-binding motifs in their carboxyl termini and that they are also putative substrates for GRKs, it is reasonable to suspect that phosphorylation of PDZ domain-binding motifs of receptors by GRK5 or other GRKs may represent a common mechanism for regulation of PDZ domain-mediated interaction in other GPCRs as well. More generally, it is also possible that other protein kinases (such as protein kinase A or PKC) may play similar roles.

The PDZ domain-mediated assembly and turnover of receptor signaling complexes is a dynamic process that is likely to be highly regulated. The regulatory mechanism involving phosphorylation of receptor might be important for receptor functions such as receptor internalization. Agonist-induced receptor internalization, a process in which activated receptors move from the cell surface into intracellular vesicles, is a common feature of GPCRs and is important for receptor regulation (33). Since many known PDZ domain-mediated protein/protein interactions are involved in the anchoring and targeting of proteins in specific membrane areas (such as synapses), it is likely that PDZ domain interaction with GPCRs will inhibit receptor internalization. PDZ domain-mediated PSD-95/β2AR interaction might be involved in anchoring the receptors in the postsynaptic membrane. Indeed, PSD-95 interaction with β2AR decreased agonist-induced receptor internalization, possibly due to the time required for the dissociation of the PSD-95/β2AR complex. When activated by agonist stimulation, GRK5 can phosphorylate agonist-bound receptors (28). This phosphorylation of the receptor by GRK5 decreases the binding affinity of PSD-95, releasing the receptor from the PSD-95 complex and facilitating internalization. Release of receptor from PSD-95 may also make it available for distinct protein interactions and signaling events. A similar regulatory mechanism may also be applicable to other proteins, such as AMPA receptors, in which phosphorylation of the glutamate receptor 2 subunit by PKC is very important in regulation of AMPA receptor internalization during synaptic plasticity (35).

Even though the evidence here strongly supports the idea that phosphorylation of β1AR decreases its association with PDZ domain-containing proteins, we cannot rule out the possibility that receptor phosphorylation might also be critical for recruiting proteins important for receptor signal transduction. In this case, phosphorylation of the receptor may serve as an initial signal to assemble receptor signaling complexes. For example, it has been reported that PKC activation can increase the phosphorylation of the glutamate receptor 2 subunit and recruit PICK1 to the excitatory synapses (35). Furthermore, cerebellar long-term depression also requires PKC-regulated interactions between the glutamate receptor 2/3 subunit and PDZ domain-containing proteins (40). Using synthetic peptides derived from the tumor suppressor MMAC/PTEN PDZ-binding domain to coprecipitate proteins from human cell lysates, Adey et al. (41) reported that threonine phosphorylation of this PDZ-binding domain both inhibits and stimulates PDZ binding.
In summary, we have provided strong evidence that GRK5, but not GRK2, can regulate the association of PSD-95 with βAR by phosphorylation of the receptor. GRK-mediated receptor phosphorylation may provide a common regulatory mechanism for the association of other GPCRs with PDZ domain-containing proteins. This regulation may be involved in many important processes such as receptor internalization, receptor localization to specific domains (e.g., synapses), and receptor signaling events.

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