Protein Kinase A Phosphorylation of Spinophilin Modulates Its Interaction with the $\alpha_{2A}$-Adrenergic Receptor (AR) and Alters Temporal Properties of $\alpha_{2A}$AR Internalization*

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Spinophilin plays critical roles in regulating trafficking and signaling of the $\alpha_{2A}$-adrenergic receptor (AR) both in vitro and in vivo (Wang, Q., Zhao, J., Brady, A. E., Feng, J., Allen, P. B., Lefkowitz, R. J., Greengard, P., and Limbird, L. E. (2004) Science 304, 1940–1944). In the present study, we demonstrate that protein kinase A (PKA) phosphorylation of spinophilin modulates the spinophilin-$\alpha_{2A}$AR interaction to regulate $\alpha_{2A}$AR internalization. Activation of PKA by forskolin abolishes the agonist-enhanced interaction between spinophilin and the $\alpha_{2A}$AR, and this event can be blocked by Ser $\rightarrow$ Ala mutations at the PKA phosphorylation sites of spinophilin. In addition, a Ser $\rightarrow$ Asp mutation that mimics the phosphorylated state at the PKA phosphorylation site Ser-177, which is located within the $\alpha_{2A}$AR binding region of spinophilin, is sufficient to block the spinophilin-$\alpha_{2A}$AR interaction in intact cells. In cells expressing mutant spinophilin carrying the S177D mutation, agonist-induced internalization of the $\alpha_{2A}$AR is accelerated and enhanced, as revealed by both intact cell enzyme-linked immunosorbent assay and quantitative immunofluorescent studies. Furthermore, activation of PKA by forskolin enhances agonist-induced internalization of the $\alpha_{2A}$AR in cells expressing wild type spinophilin but not in cells lacking spinophilin or expressing the spinophilin mutant Sp177D. These results strongly support that PKA phosphorylation of spinophilin is functionally relevant in regulating $\alpha_{2A}$AR trafficking. Therefore, modulation of spinophilin-receptor interaction through phosphorylation of spinophilin may represent a novel mechanism whereby PKA regulates G protein-coupled receptor trafficking.

The $\alpha_{2}$ adrenergic receptor (AR) belongs to the G protein-coupled receptor superfamily and couples to the $G_{i/o}$ subfamily of G proteins. In native cells, activation of the $\alpha_{2}$AR leads to inhibition of adenylyl cyclase and voltage-gated Ca$^{2+}$ channels and activation of inward rectifying K$^{+}$ channels and mitogen-activated protein kinases (1, 2). Ligand stimulation also causes internalization of the $\alpha_{2}$AR, a process that is important in regulating the sensitivity and duration of receptor-mediated signaling (3–5). $\alpha_{2}$AR internalization is mediated by $\beta$-arrestins (6), which bind to the $\alpha_{2}$AR (7–9) after the receptor is phosphorylated by G protein-coupled receptor kinases (10, 11). In previous studies, we identified spinophilin as a competitor of G protein-coupled receptor kinase and $\beta$-arrestin 2 for binding to the third intracellular loop (3iloop) of the $\alpha_{2}$AR (9, 12) and showed that interaction of spinophilin with the $\alpha_{2}$AR is enhanced by agonist stimulation of the receptor (13). As a result, spinophilin stabilizes the $\alpha_{2}$AR at the cell surface, as evident in cells without spinophilin expression, where internalization of the $\alpha_{2}$AR is significantly accelerated and enhanced (12, 14). In addition to all three $\alpha_{2}$AR subtypes ($\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$AR) (13, 14), spinophilin interacts with several other G protein-coupled receptors, including the D2 dopamine receptor (15) and the $\alpha_{2}$AR (16).

Spinophilin is a multidomain protein containing an actin binding domain (amino acids aa 1–151), a protein phosphatase 1 binding sequence (aa 427–470), a PDZ domain (aa 496–586), and three coiled-coil domains at the C terminus (aa 607–817) (17, 18). The $\alpha_{2}$AR interaction region of spinophilin is mapped to aa 151–444, which is adjacent to the actin binding domain (13). Spinophilin is a substrate of several protein kinases, including PKA (19), CaMKII (20), and ERK (21), suggesting that activities of spinophilin can be regulated by multiple intracellular pathways. PKA phosphorylation of spinophilin can be detected both in cultured cells and in the brain, and the target sites were mapped at Ser-94 and Ser-177 (19). In the present study, we addressed whether phosphorylation of spinophilin by PKA affects its interaction with the $\alpha_{2A}$AR and investigated the functional relevance of phosphorylation of spinophilin at Ser-177 on the temporal properties of $\alpha_{2A}$AR internalization. Our studies suggest that PKA regulates $\alpha_{2A}$AR trafficking through phosphorylation of spinophilin and subsequent blockade of the spinophilin-$\alpha_{2A}$AR interaction, which represents a potential novel mechanism by which other intracellular pathways regulate $\alpha_{2A}$AR function.

**EXPERIMENTAL PROCEDURES**

**Materials**

Dulbecco’s modified Eagle’s medium (Invitrogen), fetal bovine serum, Lipofectamine 2000, and calcium phosphate transfection kit were purchased from Invitrogen. Restriction
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enzymes and T4 ligase were from New England Biolab. O-Phe-nylendiamine dihydrochloride was from Pierce. Dodecyl-β-maltoside was from Calbiochem. QuikChange II site-directed mutagenesis kit was from Stratagene. Rat anti-HA monoclonal antibody, mouse anti-GFP monoclonal antibody, and immobilized protein G-agarose were from Roche Applied Science. Mouse HA.11 monoclonal antibody was from Covance. Mouse anti-Myc monoclonal antibody was from Clontech. Rabbit anti-spinophilin polyclonal antibody was from Upstate. TnT in vitro translation kit was from Promega. [35S]Methionine and [32P]orthophosphate were from GE Healthcare. All other chemicals were from Sigma-Aldrich or Fisher.

Plasmid Constructs

Construction of pCMV4-Myc-Sp94A,177A—Ser to Ala mutations were introduced at Ser-94 and Ser-177 by site-directed mutagenesis using the QuikChange II site-directed mutagenesis kit (Stratagene) following the manufacturer’s instruction. The template used was pCMV4-Myc-spinophilin (22), and mutagenesis primers used for generating 94A and 177A were 5′-CGGCGTGCGCTTGCCTGCGGCGGCGG-3′ and 5′-CGACGGCGCGCAGCTGACGGGGCGG-3′, respectively. The entire coding region of spinophilin in the final construct was sequenced at the Genomic Core Facility of University of Alabama (UAB), Heflin Center for Human Genetics. Introduced mutations were confirmed, and no additional mutations were identified.

Construction of pLEGFP-Sp177D—Ser to Asp mutation was introduced at Ser-177 by site-directed mutagenesis using the QuikChange II site-directed mutagenesis kit (Stratagene). The template used was pLEGFP-spinophilin (pLEGFP-SpWT) (23), and the mutagenesis primer used was 5′-CGACGGACGGCGGAGCTGACGGGGCGG-3′. Sequencing of the entire coding region of spinophilin in the final construct was performed at the Genomic Core Facility of UAB, Heflin Center for Human Genetics. Introduced mutations were confirmed, and no additional mutations were introduced. Sequencing was performed at the Genomic Core Facility of UAB, Heflin Center for Human Genetics.

Construction of GST-Sp151–444(177D)—Spinophilin region aa 151–444 carrying the S177D mutation was amplified by PCR using pLEGFP-Sp177D as a template and cloned into pGEX2T vector.

METHODS

Cell Culture—Immortalized mouse embryo fibroblasts (MEFs) were derived from WT (Sp+/+ or spinophilin knock-out (Sp−/−)) mice with identical genetic background (23). CosM6 cells, BOSC 293 cells, and MEFs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C/5% CO₂.

Coimmunoprecipitation of Spinophilin with the α2AAR—CosM6 cells were transfected with pCMV4-HA-α2AAR (0.3 μg/100-mm plate) together with pCMV4-Myc-SpWT or pCMV4-Myc-Sp94A,177A or pLEGFP-Sp177D (6 μg/100-mm plate) by Lipofectamine 2000 (Invitrogen). 48 h post-transfection, cells were treated with 10 μM forskolin or vehicle for 15 min and then stimulated with 100 μM epinephrine or vehicle for 3 min. Co-immunoprecipitation assays were performed as described previously (9).

Intact Cell Protein Phosphorylation—Phosphorylation of HA-α2AAR and Myc-spinophilin in intact cells was examined as described previously (9). In brief, CosM6 cells transiently transfected with cDNA encoding HA-α2AAR or Myc-SpWT were labeled with 0.1 mCi/ml [32P]orthophosphate for 1 h. Cells were then treated with 10 μM forskolin or vehicle for 15 min. HA-α2AAR or Myc-SpWT was immunosolated from cell lysates using a rat anti-HA antibody or a mouse anti-Myc antibody. Samples were separated on SDS-PAGE and analyzed by autoradiography and Western analysis.

In Vitro GST Pulldown Assay—Interaction of the α2AAR 3iloop with GST-Sp151–444 or GST-Sp151–444(177D) was examined by GST pulldown assays. Preparation of GST fusion proteins, synthesis of [35S]-labeled α2AAR 3iloop, and pulldown assays were performed as described before (9). 25 μg of GST or 2.5 μg of GST-Sp151–444 or GST-Sp151–444(177D) and an equivalent amount of [35S]-α2AAR 3iloop were used in each reaction.

Packaging of Retroviral Constructs and Transduction of MEFs—BOSC 293 cells were transfected with pLEGFP vector, pLEGFP-SpWT, or pLEGFP-Sp177D using calcium phosphate transfection kit (Invitrogen) following the manufacturer’s protocol. 48 h post-transfection, supernatants were harvested and used to transduce MEFs as described previously (23). 72 h post-transduction, MEFs stably expressing GFP or GFP-fused proteins were selected by fluorescence-activated cell sorting in the UAB Flow Cytometry Core Facility.

Antibody Labeling and Quantitative Fluorescence Analyses—To specifically detect trafficking of cell surface HA-α2AAR, MEFs cultured on coverslips were incubated with HA.11 antibody for 12 min at room temperature, followed by extensive wash to remove unbound antibody. Cells were then stimulated at 37 °C with 100 μM epinephrine or vehicle (together with 1 μM prazosin to block α1AARs and 1 μM propranolol to block βARs) for various time periods. Stimulation was stopped by putting coverslips into ice-cold PBS followed by fixation with 4% paraformaldehyde for 15 min. After three washes with PBS-0.1% Triton X-100 (PBST), coverslips were blocked with 2% bovine serum albumin in PBST for 1 h and then incubated with Alexa Fluor 594 conjugated anti-mouse secondary antibody for 1 h at room temperature. Coverslips were then washed three times with PBST and mounted for microscopy. Fluorescent images were taken using a Leica confocal microscope in the UAB High Resolution Imaging Facility and quantified using MetaMorph software. Relative internalization of HA-α2AAR was measured as internal/total fluorescent intensity normalized to untreated controls. Three independent experiments were performed, and at least 15 cells were analyzed for each time point in each experiment.

Intact Cell Surface ELISA—Intact cell surface ELISA was performed as described previously (14) to examine internalization of HA-α2AAR in response to agonist stimulation for various time periods.

Statistical Analysis—Student’s t tests (2-tail, unpaired) were performed to determine the difference between two experimental groups. p < 0.05 was considered statistically significant.
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**RESULTS**

Forskolin Treatment Eliminates Agonist-promoted Association of Spinophilin and the α2AAR—Spinophilin has been shown to be a PKA substrate in both cultured cells and murine brains (19). We first addressed whether activation of the PKA pathway would affect the spinophilin-α2AAR interaction. Forskolin has been widely used to activate PKA through elevating intracellular cAMP levels. In a previous study, forskolin was used to induce spinophilin phosphorylation at its PKA target sites (19). Cells were pretreated with 10 μM forskolin (or vehicle) and then stimulated with 100 μM epinephrine (an α2-agonist). As we demonstrated previously, agonist stimulation enhances interaction of spinophilin and the α2AAR (13) (Fig. 1A). Strikingly, forskolin pretreatment abolishes this agonist-enhanced interaction between Myc-tagged spinophilin and HA-tagged α2AAR in immunoprecipitation assays (Fig. 1A). This conclusion is confirmed by quantitative analysis (Fig. 1B). Similarly, preincubation of cells with another PKA activator, dibutyryl cAMP, also blocks the agonist-enhanced spinophilin-α2AAR interaction (data not shown). These data suggest that activation of PKA eliminates agonist-enhanced spinophilin-α2AAR association in intact cells.

Forskolin Treatment Enhances Phosphorylation of Spinophilin, but Not the α2AAR, in Intact Cells—Co-incubation of purified α2AAR with the PKA catalytic subunit leads to receptor phosphorylation in vitro (24). Therefore, forskolin treatment may lead to α2AAR phosphorylation by PKA and thereby affect its interaction with spinophilin. To test this possibility, we examined whether the α2AAR can be phosphorylated in response to forskolin treatment in intact cells. As shown in Fig. 2A, no apparent phosphorylation of the α2AAR could be detected either with or without 10 μM forskolin treatment, even after 24 h of exposure in autoradiography. By contrast, as reported previously (19), the same forskolin treatment markedly enhanced the phosphorylation level of spinophilin, which was readily detectable by autoradiography after only 2 h of exposure (Fig. 2B). Quantitative data revealed a significant enhancement of spinophilin phosphorylation in response to forskolin treatment (Fig. 2C). From these data, we conclude that in intact cells forskolin treatment enhances phosphorylation of spinophilin, but not the α2AAR.

**Phosphorylation of Spinophilin at the PKA Target Sites Is Required for Forskolin Blockade of the Spinophilin-α2AAR Interaction**—PKA phosphorylation of spinophilin primarily occurs at Ser-94 and Ser-177 (19). To test whether forskolin-induced blockade of the spinophilin-α2AAR interaction is due to PKA phosphorylation of spinophilin at these sites, we generated a Myc-tagged mutant spinophilin carrying Ala at aa 94 and aa 177 (designated Myc-Sp94A,177A, Fig. 3A). We first confirmed that Myc-Sp94A,177A cannot be effectively phosphorylated in response to PKA activation by forskolin (Fig. 3B) and then examined the interaction of the α2AAR with this spinophilin mutant. We found that Myc-Sp94A,177A was able to interact with HA-α2AAR in an agonist-promoted manner (Fig. 3C) and that the level of enhancement of the Myc-Sp94A,177A-HA-α2AAR interaction by agonist stimulation was comparable with that of the Myc-SpWT-HA-α2AAR interaction (Fig. 3D). As shown previously (Fig. 1), forskolin pretreatment abolishes co-immunoprecipitation of the HA-α2AAR with Myc-tagged WT spinophilin (Myc-SpWT) in response to agonist (p < 0.05); however, such treatment does not significantly alter agonist-enhanced association of HA-α2AAR with the Myc-Sp94A,177A mutant (p =

![FIGURE 1. Forskolin treatment blocks agonist-enhanced spinophilin-α2AAR interaction.](image1)

![FIGURE 2. Forskolin treatment enhances phosphorylation of spinophilin, but not the α2AAR, in intact cells.](image2)

**REFERENCES**

1. For example, see ref. 19.
2. mkg.
3. **, p < 0.01; n.s., not significant.
4. Mean fold change ± S.E., n = 3. **, p < 0.01. n.s., not significant.
5. Mean fold change ± S.E., n = 3. **, p < 0.01. n.s., not significant.
6. Mean fold change ± S.E., n = 3. **, p < 0.01. n.s., not significant.
7. Mean fold change ± S.E., n = 3. **, p < 0.01. n.s., not significant.
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A Ser to Asp mutation at spinophilin aa 177 disrupts the spinophilin-\(\alpha_{2A}\)AR interaction. A scheme of spinophilin structure used in co-immunoprecipitation and GST pulldown assays. B, loss of agonist-enhanced association between the HA-\(\alpha_{2A}\)AR and GFP-Sp177D mutant in intact cells. Representative blots of co-immunoprecipitation assays performed in CosM6 cells transiently expressing HA-\(\alpha_{2A}\)AR with GFP-SpWT or GFP-Sp177D. Similar results were obtained in three independent experiments. C, the receptor binding region of spinophilin (Sp151–444) carrying a Ser to Asp mutation at aa 177 exhibits reduced binding to the \(\alpha_{2A}\)AR 3iloop. In vitro GST pulldown assay was performed using GST-Sp151–444WT or Sp151–444(177D) and \(35^S\)-labeled \(\alpha_{2A}\)AR 3iloop. Similar results were obtained in three independent experiments. Upper, autoradiography of \(35^S\)-\(\alpha_{2A}\)AR 3iloop pulled down by glutathione-agarose in the presence of GST, GST-Sp151–444WT, or GST-Sp151–444(177D). Input lane represents the total amount of \(35^S\)-\(\alpha_{2A}\)AR 3iloop added in each reaction. Lower, Coomassie blue staining of GST, GST-Sp151–444WT, or GST-Sp151–444(177D) in the reaction.

FIGURE 4. A Ser to Asp mutation at spinophilin aa 177 disrupts the spinophilin-\(\alpha_{2A}\)AR interaction. A, scheme of spinophilin structure used in co-immunoprecipitation and GST pulldown assays. B, loss of agonist-enhanced association between the HA-\(\alpha_{2A}\)AR and GFP-Sp177D mutant in intact cells. Representative blots of co-immunoprecipitation assays performed in CosM6 cells transiently expressing HA-\(\alpha_{2A}\)AR with GFP-SpWT or GFP-Sp177D. Similar results were obtained in three independent experiments. C, the receptor binding region of spinophilin (Sp151–444) carrying a Ser to Asp mutation at aa 177 exhibits reduced binding to the \(\alpha_{2A}\)AR 3iloop. In vitro GST pulldown assay was performed using GST-Sp151–444WT or Sp151–444(177D) and \(35^S\)-labeled \(\alpha_{2A}\)AR 3iloop. Similar results were obtained in three independent experiments. Upper, autoradiography of \(35^S\)-\(\alpha_{2A}\)AR 3iloop pulled down by glutathione-agarose in the presence of GST, GST-Sp151–444WT, or GST-Sp151–444(177D). Input lane represents the total amount of \(35^S\)-\(\alpha_{2A}\)AR 3iloop added in each reaction. Lower, Coomassie blue staining of GST, GST-Sp151–444WT, or GST-Sp151–444(177D) in the reaction.

FIGURE 3. Phosphorylation of spinophilin Ser-94 and Ser-177 is required for blockade of the agonist-enhanced spinophilin-\(\alpha_{2A}\)AR interaction by forskolin. A, scheme of spinophilin structure. Ser-94 and Ser-177 are the two major PKA phosphorylation sites. In Sp94A,177A, these two sites were mutated to Ala. B, forskolin treatment fails to promote phosphorylation of the Myc-Sp94A,177A mutant. Left, representative images from autoradiograph and Western blot of Myc-Sp94A,177A. Right, quantitative data of fold change in spinophilin phosphorylation in response to forskolin treatment. Values represent mean \(\pm\) S.E. \(n = 3\), not significant. C, representative blots of co-immunoprecipitation assays. CosM6 cells transiently transfected by HA-\(\alpha_{2A}\)AR together with Myc-SpWT or Myc-Sp94A,177A were pretreated with forskolin or vehicle for 15 min and then stimulated with epinephrine for 3 min. D, quantitative data of agonist-induced changes (no agonist added defined as 1.0-fold) of Myc-SpWT or Myc-Sp94A,177A associated with HA-\(\alpha_{2A}\)AR with or without forskolin. Values represent mean \(\pm\) S.E. \(n = 4\). *\(p < 0.05\), n.s., not significant.

0.39) (Fig. 3, C and D). These data suggest that phosphorylation of spinophilin at the PKA target sites Ser-94 and Ser-177 is required for blockade of the spinophilin-\(\alpha_{2A}\)AR interaction by forskolin.

A Ser to Asp Mutation at Spinophilin aa 177 Is Sufficient to Cause Loss of Interaction of Spinophilin with the \(\alpha_{2A}\)AR in Intact Cells—Ser-177 is located within the region of spinophilin that directly binds to the \(\alpha_{2A}\)AR 3iloop (13) (Fig. 4A). We therefore addressed whether phosphorylation of spinophilin at Ser-177 is sufficient to alter interaction of spinophilin with the \(\alpha_{2A}\)AR. We generated a GFP-tagged mutant spinophilin carrying Asp at aa 177 (designated GFP-Sp177D, Fig. 4A), which mimics the phosphorylated state at this site by introducing a negative charge, and examined its interaction with the HA-\(\alpha_{2A}\)AR in response to agonist stimulation. Although agonist treatment enhances association of GFP-tagged WT spinophilin (GFP-SpWT) with HA-\(\alpha_{2A}\)AR, it fails to promote interaction of the receptor with GFP-Sp177D (Fig. 4B), suggesting that mimicking the phosphorylated state at Ser-177 of spinophilin is sufficient to cause the loss of agonist-enhanced spinophilin-\(\alpha_{2A}\)AR interaction in intact cells.

We further tested whether the S177D mutation affects direct interaction between spinophilin aa 151–444 (Sp151–444) and the \(\alpha_{2A}\)AR 3iloop. We generated GST-fused mutant Sp151–444 carrying Asp at aa 177 (GST-Sp151–444(177D), Fig. 4A) and examined its interaction with the radiolabeled \(\alpha_{2A}\)AR 3iloop by pulldown assays. Compared with GST-Sp151–444WT, the GST-Sp151–444(177D) mutant has a remarkably reduced interaction with the \(\alpha_{2A}\)AR 3iloop (Fig. 4C), suggesting that mimicking the phosphorylated state at Ser-177 reduces the direct interaction between spinophilin and the \(\alpha_{2A}\)AR.
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**FIGURE 5.** Spinophilin carrying the S177D mutation has reduced membrane localization. Sp−/− MEFs stably expressing HA-α2AR were transduced with retrovirus encoding GFP alone, GFP-SpWT, or GFP-Sp177D. A, representative images of GFP, GFP-SpWT, and GFP-Sp177D expressed in Sp−/− MEFs. B, quantification of percentage of green fluorescence on plasma membrane in MEFS expressing GFP-SpWT or GFP-Sp177D. Values represent mean ± S.E. n = 23. **, p < 0.01.

**FIGURE 6.** Intact cell ELISAs reveal accelerated turnover of HA-α2AR from the surface of GFP-Sp177D-expressing Sp−/− cells compared with WT or GFP-SpWT-expressing Sp−/− cells. A, total cell lysates of Sp−/− cells or Sp−/− cells stably expressing HA-α2AR with GFP alone, GFP-SpWT, or GFP-Sp177D were separated by SDS-PAGE and blotted with anti-spinophilin antibody to show endogenous spinophilin or exogenous GFP-SpWT or GFP-Sp177D expression (Upper). Actin in cell lysates is shown as loading controls (Lower). B, intact cell surface ELISAs performed with different types of cells. Values represent mean ± S.E. n = 12 for each time point in each type of cell. **, p < 0.01.

Mutant Spinophilin Carrying 177D Exhibits Reduced Localization at the Plasma Membrane—It has been reported that phosphorylation at Ser-177 attenuates spinophilin interaction with actin (19), which plays an important role in targeting spinophilin to cellular compartments, including the plasma membrane (23). Therefore, we compared the cellular distribution of GFP, GFP-SpWT, and GFP-Sp177D. Both GFP-SpWT and GFP-Sp177D, but not GFP alone, can be detected at the plasma membrane and in cytosolic compartments (Fig. 5A). Quantitative analysis revealed that ~55% of GFP-SpWT and 38% of GFP-Sp177D are localized at the plasma membrane (Fig. 5B), suggesting a reduction in surface localization of spinophilin caused by mimicking the phosphorylated state at Ser-177. The reduction is statistically significant (p < 0.01). Because the α2AR is localized at the cell surface, reduced localization of Sp177D at this compartment would also contribute to loss of interaction between the α2AR and this spinophilin mutant in intact cells.

Agonist-induced Internalization of the α2AR Is Accelerated in Cells Expressing Sp177D—To further understand the functional relevance of phosphorylation of spinophilin at Ser-177 on α2AR trafficking and signaling, we evaluated the impact of the S177D mutant spinophilin on the rate of α2AR internalization. We used retroviral transduction to introduce GFP, GFP-SpWT, or GFP-Sp177D into Sp−/− MEFS stably expressing HA-α2AR. Cells stably co-expressing HA-α2AR with GFP or GFP-tagged proteins were selected by fluorescence-activated cell sorting for use in the following experiments. Western analysis confirmed that exogenous GFP-SpWT or GFP-Sp177D was expressed in Sp−/− cells at levels comparable with that of endogenous spinophilin in Sp+/+ MEFS (Fig. 6A). We compared α2AR internalization in Sp−/− cells expressing different GFP fusion proteins with that in Sp+/+ cells by intact cell surface ELISA. Consistent with our previous report (14), internalization of α2AR is accelerated and enhanced in Sp−/− cells as compared with that in Sp+/+ cells (Fig. 6B). Reintroduction of GFP-SpWT into Sp−/− cells completely rescues this phenotype, whereas expression of GFP-Sp177D in Sp−/− cells fails to do so (Fig. 6B). These data suggest that mimicking the phosphorylated state at Ser-177 of spinophilin leads to acceleration and enhancement of agonist-induced internalization of the α2AR.

We also exploited quantitative immunofluorescent studies to examine redistribution of cell surface α2AR in response to agonist stimulation in Sp−/− cells expressing GFP, GFP-SpWT, or GFP-Sp177D. Cell surface HA-α2ARs were first labeled with an anti-HA antibody and then stimulated by agonist for various time periods. The receptor-antibody complexes were internalized in response to agonist treatment and visualized using a fluorescent conjugated secondary antibody and confocal microscopy. In cells expressing GFP alone or GFP-Sp177D, 10 min of treatment with epinephrine caused internalization of surface HA-α2AR to the perinuclear region, whereas in cells expressing GFP-SpWT or GFP-Sp177D expressing HA-α2AR were transduced with retrovirus encoding GFP alone, GFP-SpWT, or GFP-Sp177D. A, representative images of GFP, GFP-SpWT, and GFP-Sp177D expressed in Sp−/− MEFS. B, quantification of percentage of green fluorescence on plasma membrane in MEFS expressing GFP-SpWT or GFP-Sp177D. Values represent mean ± S.E. n = 23. **, p < 0.01.

**FIGURE 5.** Spinophilin carrying the S177D mutation has reduced membrane localization. Sp−/− MEFs stably expressing HA-α2AR were transduced with retrovirus encoding GFP alone, GFP-SpWT, or GFP-Sp177D. A, representative images of GFP, GFP-SpWT, and GFP-Sp177D expressed in Sp−/− MEFS. B, quantification of percentage of green fluorescence on plasma membrane in MEFS expressing GFP-SpWT or GFP-Sp177D. Values represent mean ± S.E. n = 23. **, p < 0.01.
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A

HA-α2AR in cells expressing

| Ag | GFP | GFP-SpWT | GFP-Sp177D |
|----|-----|---------|-----------|
| 0’ | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| 10’ | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| 30’ | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |

**FIGURE 7. Agonist-induced internalization of the HA-α2AR occurs to a greater extent in cells expressing GFP-Sp177D than in cells expressing GFP-SpWT. Sp+ MEFs stably expressing HA-α2AR with GFP alone, GFP-WT spinophilin, or GFP-Sp177D were stimulated with 100 μM epinephrine (together with 1 μM prazosin to block α1AR) and 1 μM propranolol to block βAR) for various times. A, representative images showing distribution of HA-α2AR at different time points. B, quantification of fluorescent images of HA-α2AR revealing relative internalization of HA-α2AR in response to agonist stimulation. Values represent mean ± S.E. n = 3 independent experiments, and 15–18 cells were analyzed for each time point in each experiment. *, p < 0.05. **, p < 0.01. n.s., not significant.**

expressing GFP-SpWT, the majority of HA-α2AR was still localized at the cell surface at this time point (Fig. 7A). After 30 min of agonist treatment, internalization of α2AR to the perinuclear area could be readily detected in all cell types (Fig. 7A). Quantification of receptor fluorescence revealed that internalization of the HA-α2AR in cells expressing GFP-Sp177D occurs at a level similar to that observed in cells expressing GFP alone, which is significantly higher than that in cells expressing GFP-SpWT at both 10- and 30-min time points (Fig. 7B). These data provide additional evidence that α2AR internalization is accelerated and enhanced by expression of spinophilin carrying the S177D mutation.

Forskolin Treatment Accelerates Agonist-induced Internalization of the α2AR in Cells Expressing WT Spinophilin, but Not in Cells Lacking Spinophilin or Expressing Sp177D—The studies described above demonstrate that a spinophilin mutation mimicking the phosphorylated state at one of the PKA target sites, Ser-177, leads to alteration of α2AR trafficking. We sought to directly address whether activation of the PKA pathway changes the temporal properties of agonist-induced α2AR internalization and whether the phosphorylation state of spinophilin is important in this process. Therefore, we examined α2AR internalization in Sp−/− cells expressing GFP alone or GFP-tagged SpWT or Sp177D with or without forskolin pretreatment using a quantitative immunofluorescent approach. In the absence of forskolin, we again observed accelerated α2AR internalization in cells expressing GFP alone or GFP-Sp177D as compared with that in cells expressing GFP-SpWT (Fig. 8, A, C, and E, left panels). In cells expressing GFP-SpWT, forskolin pretreatment dramatically enhanced agonist-induced internalization of the α2AR at both 10- and 30-min time points (Fig. 8, C and D), indicating that activation of the PKA pathway accelerates and enhances agonist-induced internalization of the α2AR in these cells. By contrast, in cells expressing GFP alone (Fig. 8, A and B) or GFP-Sp177D (Fig. 8, E and F), forskolin pretreatment did not lead to significant changes in α2AR internalization as compared with the no-treatment control, demonstrating the importance of spinophilin and its phosphorylation state in PKA-mediated regulation of α2AR trafficking. Taken together, these data suggest that activation of PKA indeed accelerates and enhances agonist-induced α2AR trafficking and that phosphorylation of spinophilin at the PKA target site, Ser-177, is critical for such regulation.

DISCUSSION

PKA is an important intracellular Ser/Thr kinase involved in numerous cellular processes. PKA can directly phosphorylate some G protein-coupled receptors, including β1 (25, 26) and β2AR (27, 28), M1 muscarinic acetylcholine receptor (29), D1 dopamine receptor (30), and chemokine receptor (31). PKA phosphorylation of these receptors induces heterologous desensitization (25–31) and receptor internalization (29, 32), leading to reduced responsiveness to agonist stimulation. Although the α2AR can be phosphorylated by the PKA catalytic subunit in vitro (24), we failed to detect phosphorylation of α2AR in response to forskolin treatment in intact cells despite repetitive trials (Fig. 2A), suggesting that the α2AR may not be a direct target for PKA-mediated regulation in cells. Instead, the present study has revealed a potential novel mechanism by which PKA regulates α2AR. We showed that activation of PKA by forskolin treatment attenuates the interaction between α2AR and spinophilin and thereby alters α2AR trafficking.
Phosphorylation Modulates the Spinophilin-α2A-AR Interaction

Our further studies suggest that PKA phosphorylation of spinophilin is required and sufficient for this regulation. Spinophilin has two major PKA phosphorylation sites, Ser-94 and Ser-177. Mutation of these two sites to Ala leads to loss of forskolin blockade of the agonist-enhanced spinophilin-α2A-AR interaction (Fig. 3, C and D), suggesting that phosphorylation of spinophilin at the PKA target sites is required for this process. Ser-177 is located within the region of spinophilin (Sp151–444) that directly interacts with the α2A-AR 3loop (13). A Ser to Asp mutation at this site, which mimics the phosphorylated state by introducing negative charge, remarkably attenuated direct binding of Sp151–444 to the α2A-AR 3loop (Fig. 4C). In addition, full-length spinophilin carrying the 177D mutation exhibits a significantly reduced plasma membrane localization (Fig. 5B), presumably due to attenuated association with actin (19). Therefore, we conclude that the inability of α2-agonist to promote interaction of the α2A-AR with Sp177D in cells (Fig. 4B) is due to a decreased ability of this spinophilin mutant to bind to the α2A-AR as well as its altered cellular distribution.

In cells expressing the Sp177D mutant, agonist-induced internalization of the α2A-AR is enhanced and accelerated (Figs. 6 and 7), conceivably due to loss of the spinophilin-α2A-AR interaction and spinophilin-dependent regulation. Indeed, the kinetics of α2A-AR internalization in cells expressing Sp177D resembles that observed in Sp−/− cells (Fig. 6). These data suggest that PKA phosphorylation of spinophilin is functionally relevant in regulating α2A-AR trafficking. In further support of this notion, we found that activation of PKA accelerates and enhances agonist-induced α2A-AR internalization in cells expressing WT spinophilin, but not in cells lacking spinophilin or expressing the Sp177D mutant (Fig. 8).

In addition to PKA, spinophilin is a substrate of several other kinases, including CaMKII (20) and ERK (21). Whether phosphorylation of spinophilin by these kinases also affects spinophilin interaction with α2A-AR remains to be determined. In summary, this study suggests that modulation of the spinophilin-α2A-AR interaction represents a potential novel pathway where cross-talk between G protein-coupled receptors and other intracellular signaling pathways may occur.

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FIGURE 8. Forskolin pretreatment enhances agonist-induced HA-α2A-AR internalization in cells expressing GFP-SpWT, but not in cells expressing GFP alone or GFP-Sp177D. Sp−/− MEFs stably expressing HA-α2A-AR with GFP alone (A and B), GFP-WT spinophilin (C and D), or GFP-Sp177D (E and F) were pretreated with 10 μM forskolin or vehicle for 15 min and then stimulated with epinephrine (together with 1 μM prazosin and 1 μM propranolol) for various times. A, C, E, representative images showing distribution of HA-α2A-AR at different time points. B, D, F, quantification of fluorescent images of HA-α2A-AR in A, C, E, respectively, revealing relative internalization of HA-α2A-AR in response to agonist stimulation. Values represent mean ± S.E. n = 3 independent experiments, and 12–15 cells were analyzed for each time point in each experiment. *, p < 0.05. n.s., not significant.
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