Previously, we demonstrated that the third intracellular (3i) loop of the heptahelical α2A-adrenergic receptor (α2AAR) is critical for retention at the basolateral surface of polarized Madin-Darby canine kidney II (MDCKII) cells following their direct targeting to this surface. Findings that the 3i loops of the D2 dopamine receptors interact with spinophilin (Smith, F. D., Oxford, G. S., and Milgram, S. L. (1999) J. Biol. Chem. 274, 19894–19900) and that spinophilin is enriched beneath the basolateral surface of polarized MDCK cells prompted us to assess whether α2AR subtypes might also interact with spinophilin. [35S]Met-labeled 3i loops of the α2AAR (Val18-Lys210), α2BAR (Lys210-Trp234), and α2C AR (Arg197–Val213) subtypes interacted with glutathione S-transferase-spinophilin fusion proteins. These interactions could be refined to spinophilin amino acid residues 169–255, in a region between spinophilin’s F-actin binding and phosphatase 1 regulatory domains. Furthermore, these interactions occur in intact cells in an agonist-regulated fashion, because α2AR and spinophilin coimmunoprecipitation from cells is enhanced by prior treatment with agonist. These findings suggest that spinophilin may contribute not only to α2AR localization but also to agonist modulation of α2AR signaling.

The three α2-adrenergic receptor (α2AR)1 subtypes are members of the type II, biogenic amine-binding, G protein-coupled receptor family. These receptor subtypes all couple via the Gi/Go family of GTP-binding proteins to the inhibition of adenyl cyclase, inhibition of voltage-dependent calcium channels, potentiation of potassium currents via G protein-coupled, inwardly rectifying potassium channels, activation of phospholipase D, and activation of MAP kinase in native cells (1–4). In heterologous cell systems, these receptors also couple to the activation of a variety of signaling molecules, including Ras (5–7), p70S6 kinase (8), MAP kinase (9, 10), and phospholipase D (11).

Although all three α2ARs appear to activate similar signaling pathways, differences in the cellular trafficking of these subtypes have been reported, both in naive cells and following agonist activation. Subtype-selective differences in agonist-elicted α2AR redistribution have been noted in several experimental systems (12–18). The α2m AR subtype is readily internalized following agonist activation, whereas the α2AAR subtype typically is not (14, 18). The α2C AR subtype has not been explored in as much detail with regard to agonist-elicted redistribution because of its considerable accumulation intracellularly (14). The α2AR subtypes also manifest different trafficking itineraries in polarized Madin-Darby canine kidney II (MDCKII) cells, even in the absence of agonist treatment. The α2AAR subtype is targeted directly to the basolateral surface (19), whereas the α2m AR subtype is delivered randomly to both the apical and basolateral surfaces but is selectively retained on the basolateral surface (t1/2 = 10–12 h) in contrast to its rapid loss from the apical surface (t1/2 = 5–15 min) (20). These findings suggest that there is a molecular mechanism responsible for the selective retention of the α2m AR on the basolateral sub-domain of MDCK cells, probably a retention mechanism shared by the basolaterally targeted α2AAR and α2C AR subtypes (20). Although α2ARs, like α2AARs, are directly targeted to and retained on the basolateral subdomain, a significant proportion of these receptors is identifiable in an intracellular pool at steady state (14, 18, 20); the functional relevance of this intracellular α2C AR pool has yet to be clarified.

Receptor retention on the lateral subdomain of MDCKII cells likely involves the third intracellular loop of the α2AR subtypes. For example, deletion of this loop in the α2AAR subtype (Δ3i α2AAR) results in accelerated basolateral receptor turnover (t1/2 = 4.5 h) when compared with that for the wild-type receptor or with α2AAR structures that have been mutated in the N terminus or the C-terminal tail (all possessing a t1/2 of 10–12 h) (21). Similarly, the Δ3i α2B AR is not enriched at the basolateral surface of MDCKII cells at steady state (22).

Based on our findings that the α2m AR is rapidly removed from the apical surface following random delivery and that removal of the 3i loops of the α2AAR and α2C AR subtypes accelerates surface turnover of these receptors, we hypothesize that α2ARs interact, via their 3i loops, with protein(s) enriched beneath the basolateral surface of MDCKII cells to stabilize...
their steady-state localization. Consequently, we were particularly intrigued by recent findings that the 3i loop of another G/Gi-coupled G protein-coupled receptor, the D₂ dopamine receptor, interacts with spinophilin (23–25), and that this protein is enriched beneath the basolateral surface of polarized MDCK cells (24). In addition, the multiple protein-interacting domains within spinophilin (24) suggest that its interaction with the receptor may facilitate the formation of a signaling complex to modulate signaling or recruitment of other proteins with the receptor may facilitate the formation of a signaling complex to modulate signaling or recruitment of other proteins.

Experimental Procedures

Materials—The pGEMEX-2 vector and TnT in vitro translation kit were from Promega (Madison, WI). The [35S]methionine (1000 Ci/mmol, at 10 μCi/ml) was purchased from PerkinElmer Life Sciences (Boston, MA). PVDF nylon membranes were from Millipore (Bedford, MA). The fast protein liquid chromatography and DEAE-Sephacel columns were from Amersham Pharmacia Biotech (Piscataway, NJ). Dodecyl-β-maltoside and cholesteryl-hemisuccinate were purchased from Calbiochem (San Diego, CA) and Sigma Chemical Co. (St. Louis, MO), respectively. Antibodies against the HA epitope engineered into the receptor may facilitate the formation of a signaling complex to modulate signaling or recruitment of other proteins with the receptor may facilitate the formation of a signaling complex to modulate signaling or recruitment of other proteins. Antibodies against the HA epitope engineered into the receptor may facilitate the formation of a signaling complex to modulate signaling or recruitment of other proteins with the receptor may facilitate the formation of a signaling complex to modulate signaling or recruitment of other proteins. Antibodies against the HA epitope engineered into the receptor may facilitate the formation of a signaling complex to modulate signaling or recruitment of other proteins with the receptor may facilitate the formation of a signaling complex to modulate signaling or recruitment of other proteins.

MDCK cell Culture and Polarization—MDCKII cells were plated at confluence (1–2.5 × 10⁶ cells) and grown on 12-mm Transwell filters (4.4-μm pore size, Costar, Cambridge, MA) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Sigma) and 100 units/ml penicillin and 10 μg/ml streptomycin at 37 °C/5% CO₂ as described previously (19) except with daily media changes for 5–7 days. Under these conditions, cells form a monolayer and functionally polarize with distinct apical and basolateral surfaces separated by tight junctions. We routinely verify that tight junctions have formed and that the apical and basolateral compartments are functionally separated from one another using the nontransportable molecule [3H]methoxy-inulin (19). For these leak assays, 2 μCi of [3H]methoxy-inulin is added to the apical subcompartment and incubated for 1 h at 37 °C/5% CO₂, followed by counting 100 μl of the medium in each of the apical and basolateral subcompartments. Leaks range from 5–10%, and we discard from study any culture wells of >10% leak.

Immunofluorescent Labeling and Confocal Microscopy

Antibody Purification—Rabbit anti-spinophilin antibodies were generated by injection of purified glutathione S-transferase (GST)-fusion proteins (fused to spinophilin amino acids 286–390) as described previously by MacMillan et al. (26). Antibodies were purified from serum by affinity chromatography. Affinity matrices were generated by mixing 2 ml of Affi-Gel-15 and 1 ml of Affi-Gel-10 (Bio-Rad) equilibrated in 0.1 m HEPES, pH 7.0, in a 10 ml of a Poly Prep chromatography column (Bio-Rad). Purified GST-Sp286–390 fusion protein (11.7 mg in 6.5 ml of PBS) was loaded onto the column and incubated with inversion for 4 h at 4 °C. The resin was washed with 1× PBS until free of unbound GST-Sp286–390, as determined by A₂₈₀. Unbound sites on the Affi-Gel matrix were blocked by incubation with 1 ml ethanolamine for 1 h at 4 °C with inversion. The column was equilibrated with 1× PBS (0.05% NaNO₃) and stored at 4 °C. A GST “subtraction column” was prepared in the same manner, except GST alone was coupled to the Affi-Gel 10/15 mixture matrix.

Serum (2 ml) was added to the GST-Sp286–390 affinity matrix and incubated with rotation for 2 h at room temperature. The column was washed three times with 1× PBS, once with 333 mM NaCl in 1× PBS, and then twice more with 1× PBS. Antibody was eluted twice with 2 ml of 100 mM glycine, pH 2.5, and collected into 200 μl of 1× Tris-HCl, pH 9.0, to neutralize the sample. Eluted antibody was pooled, concentrated, and exchanged into 1× PBS using an Amicon Stirred Cell with a YM30 filter (Amicon). To remove antibody directed against the GST portion of the GST-spinophilin fusion protein, concentrated antibody was incubated with the GST subtraction column, prepared as described above, by rotation for 30 min at room temperature. The pass-through from this column was collected and concentrated using an Amicon Stirred Cell as described above, and utilized as the anti-Sp286–390 antibody. Antibody concentration was determined to be 1.44 mg/ml by protein assay (Bradford). Optimal working concentrations of antibody in Western and immunolocalization were derived empirically via Western blot analysis and immunofluorescence staining.

Polarization and Immunolocalization—Polarized MDCKII cells stably expressing the individual α₂AR subtypes were grown on Transwells, as described above, and then rinsed once with PBS-CM (phosphate-buffered saline with 1 mm MgCl₂ and 0.5 mm CaCl₂) and fixed for 15 min with either 100% methanol (MeOH) at −20 °C or with 4% parafomaldehyde at room temperature (−22 °C) followed by quenching with two sequential 7.5-min incubations with 50 mM NH₄Cl in PBS-CM. Spinophilin immunolocalization was best observed under 4% methanol fixation, whereas the α₂AR localization (“signal-to-background” ratio) was best visualized following parafomaldehyde fixation and quenching. For co-localization studies, we used MeOH for fixation of the polarized MDCKII cells.

After fixation, cells were rinsed two more times in PBS-CM, permeabilized with 0.2% Triton X-100 added to the cell surface of the excised Transwell for 20 min, and incubated in blocking buffer (0.1% Triton X-100 and 2% bovine serum albumin in PBS-CM) for 1 h. Primary antibody was added to the cell side of excised Transwells and incubated for either 1 h at room temperature or overnight (15–18 h) at 4 °C. Mouse 12CA5 anti-HA antibodies were diluted at 1:250 (4 μg/ml), and rabbit anti-spinophilin 286–390 antibodies were used at a dilution of 1:100 (PBS-CM). MDCKII cells were washed three times for 15 min in PBS-CM at 22 °C before adding secondary antibodies. The secondary antibodies were Alexa488- or Cy3-conjugated anti-rabbit or anti-mouse antibodies, diluted 1:1000 (2 μg/ml) and were incubated with the cells for 1 h at room temperature. Cells were again rinsed three times for 15 min in PBS-CM and mounted cell-side-up onto a glass slide with Aquapolymount and sealed under a glass coverslip. Images were visualized on a Zeiss LSM 410, laser-scanning, confocal microscope in the Vanderbilt Cell Imaging Core Facility. Images were taken through a 40× oil objective at 1.5× magnification.

Generating [35S]Met-labeled α₂AR 3i Loops as Ligands

The residues corresponding to the 3i loops of the α₂AR (amino acids 217–377 (27i)), the α₁β₂AR (amino acids 210–354 (28i)), and the α₂AR (amino acids 248–363 (29)) were subcloned into the pGE MX2 vector in-frame within the polylinker downstream of the sequence encoding the methionine-rich co-translational signal sequence in vitro. A GST “subtraction column” was prepared in which four methionines were inserted via polymerase chain reaction into the N-terminal region of the α₂AR 3i loop ((Met³)₅α₂AR). Antibody was added to the cell side of excised Transwells and incubated for 180 min at room temperature or overnight (15–18 h) at 4 °C. Mouse 12CA5 anti-HA antibodies were diluted at 1:250 (4 μg/ml), and rabbit anti-spinophilin 286–390 antibodies were used at a dilution of 1:100 (PBS-CM). MDCKII cells were washed three times for 15 min in PBS-CM and mounted cell-side-up onto a glass slide with Aquapolymount and sealed under a glass coverslip. Images were visualized on a Zeiss LSM 410, laser-scanning, confocal microscope in the Vanderbilt Cell Imaging Core Facility. Images were taken through a 40× oil objective at 1.5× magnification.

GST-spinophilin Fusion Protein Generation

GST-spinophilin fusion proteins were generated with spinophilin amino acid regions 151–444 and 169–255 and expressed in DH5α. Bacteria were grown at 37 °C to an A₆₀₀ of 0.6. GST or GST fusion protein expression was initiated with the addition of 1 μM isopropyl-β-D-thiogalactopyranoside and allowed to proceed for 2–6 h at 37 °C. Bacteria were collected by centrifugation at 10,000 × g and then lysed in 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 1 mg/ml lysozyme, 200
mm NaCl, 100 μM PMSF, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 10 units/ml aprotinin (TT buff) by one freeze-thaw cycle followed by probe sonication for 30-3 s bursts on ice. GSH-agarose (1 ml of a 1:1 slurry equilibrated in TT buff) was added to the supernatant of a 13,000 × g centrifugation and incubated for 1 h at 4 °C with inversion. This solution was transferred to a 0.8- × 4-cm Poly-Prep column (Bio-Rad) and washed with 12 ml of TT buff, 3 ml of 333 mM NaCl in TT buff, and then with 6 ml of TT buff. GST or GST fusion protein was eluted from the GSH-agarose by adding 3 ml of 10 mM free acid GSH in TT pH 7.5. Eluted protein was concentrated and exchanged into PBS buffer using an Amicon Stirred Cell.

**Binding of 3i Loops to GST-spinophilin**

Equimolar concentrations of GST-spinophilin fusion protein were incubated with 300,000 cpm (estimated to represent ~40 pmol)[35S]Met-labeled α2A, α2C, or α2D loop ligand (see above). GST-spinophilin (1:1 slurry equilibrated with TT buff) was then added to this incubation, rotated for 2 h at 4 °C, and the resin collected by centrifugation. The resin was then exposed to four 1-ml TT washes. Interaction with GST-spinophilin versus GST (controls) was determined by elution of the 3i loop into 1× Laemmli buffer (400 mM Tris, pH 6.8, 700 mM β-mercaptoethanol, 1% SDS, 10% glycerol) and separation of the eluates by 12% SDS-PAGE. The degree of interaction was quantitated by cutting nonadsorbed proteins were removed by washing the protein A resin three times in Dj/M/CHS wash buffer (1 mg/ml DijM, 0.2 mg/ml CHS, 20% glycerol, 25 mM glycyglycine, pH 7.6, 20 mM HEPES, pH 7.6, 100 mM NaCl, 5 mM EGTA, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 10 units/ml aprotinin, and 100 μM PMSF) and centrifugation at 4 °C. Proteins were eluted with the addition of 1× Laemmli buffer and heating to 70 °C for 5 min. Eluates were separated via 10% SDS-PAGE, transferred to an Immobilon P membrane (PVDF; Millipore) with a constant current of 1 amp for 72 min in CAPS transfer buffer (1 M cyclohexylamino-1-propane sulfonic acid (CAPS), pH 11, 10% methanol), and subjected to Western blot analysis.

**Western Blot Analysis**

PVDF membranes were blocked for 15 min in Tris-buffered saline (20 mM Tris, pH 7.6, 157 mM NaCl) with 0.1% Tween 20 (TBST) and 5% Carnation Instant Powdered milk (w/v). The appropriate primary antibody was then added at a dilution of 1:1000 (Rat anti-HA) or 1:2000 (mouse anti-Myc monoclonal antibody) in blocking buffer and incubated at room temperature for 1.5–2 h. Blots were washed three times for 15 min with TBST and exposed to horseradish peroxidase-conjugated anti-rat or anti-mouse secondary antibodies, as appropriate, at a 1:2000 dilution in 5× TBST at room temperature. Blots were washed again three times for 15 min in TBST, incubated with ECL Western blotting detection reagent (Amer sham Pharmacia Biotech, Buckinghamshire, UK) for 1.5 min, and then exposed to x-ray film for variable times ranging from 5 s to 30 min.

**RESULTS**

**Communolocalization of Endogenous Spinophilin with the α2AR Subtypes in MDCKII Cells**—Spinophilin is a ubiquitously expressed multidomain protein (25) composed of an F-actin binding domain (amino acids 1–153), a PP1 binding/regulatory domain, and a C terminus that possesses a series of coiled-coil sequences similar to spinophilin’s structural homolog, neurabin I (Fig. 2A). Satoh et al. (24) showed that spinophilin was localized to the lateral sub-domain in polarized MDCKII cells. As shown previously, the α2A-adrenergic receptor also is enriched on the lateral sub-domain of these cells (19, 20) and is revealed here using a Cy3 (red signal)-conjugated secondary antibody directed against the 12CA5 antibody that recognizes the N-terminal HA epitope in the α2A-AR (Fig. 1). A rabbit polyclonal antibody was raised against amino acids 286–390 in spinophilin (26), a region that has virtually no sequence similarity to spinophilin’s structural homolog, neurabin I (Fig. 2A). As shown in Fig. 1, the affinity-purified polyclonal antibody against spinophilin, visualized here via Alexa488 (green signal)-conjugated secondary antibody, re-
veals considerable enrichment of endogenous spinophilin at the lateral surface of these polarized cells, corroborating initial reports of Satoh et al. (24). The overlap of expression of the α2AR and spinophilin in the lateral domain of MDCKII cells is demonstrated by the considerable amount of yellow signal present in the red/green overlay. Similar results were observed upon colocalization of the α2AR subtype and spinophilin (data not shown). It should be noted, however, that some spinophilin also is detected intracellularly, including in a sub-apical compartment.

The 3i Loops of All Three α2AR Subtypes Interact with Spinophilin—Smith et al. (23) have demonstrated, via yeast two-hybrid screens and gel overlay strategies, that the 3i loops of the D2 dopamine receptor (short and long forms) interact with spinophilin in the region between the F-actin binding and PP1 domains (Fig. 2A). Consequently, we created GST fusion proteins of spinophilin bounded between amino acids 151 and 444. For these assays, the radiolabeled probe was [35S]Met-(Met)4-GST-Sp151–444. Radiolabeled [35S]Met-(Met)4-Gen10 loop fusion protein retained in the GSH-agarose-GST fusion protein pellet was visualized by autoradiography and quantitated via scintillation counting. C, the α2AR-binding domain of spinophilin can be further refined to Sp169–255; virtually no interaction with Neurabin 146–453 can be observed. For these assays, the radiolabeled probe was [35S]Met-(Met)4-GST-Sp151–444. Radiolabeled [35S]Met-(Met)4-Gen10–3i loop fusion protein retained in the GSH-agarose-GST fusion protein pellet was visualized by autoradiography and quantitated via scintillation counting. The amount of [35S]Met-(Met)4-Gen10–3i loop added to each of the GST fusion protein binding incubations was selected because it represents the region of least homology of GST-spinophilin 151–444. Furthermore, binding to the 3i loop is demonstrated by the more restricted region of Sp169–255; in fact, binding to the α2-3i loop to this region is more readily detected to this region, than to GST-Sp151–444.

Interaction of the α2AR and Spinophilin within the Cell Is Regulated by Agonist—It was of interest to determine whether or not these α2AR-3i loop-spinophilin interactions, detected in vitro via GST fusion protein assays, could be detected in the context of a living cell. For these studies, we transiently coexpressed cDNAs encoding full-length HA-tagged α2ARs in CosM6 cells. On the day of analysis, cells were incubated with or without 100 μM epinephrine prior to extraction of the cell membranes and solubilization with D2M/CHS, a detergent that extracts receptor in a functional conformation (30). As shown in Fig. 3, immunoprecipitation of Myc-tagged spinophilin leads to the coisolation of the HA-α2AR in cells expressing both receptor and spinophilin (lanes 2 and 3; lane 1 is an extract from CosM6 cells expressing only the HA-tagged α2AR). HA-tagged α2AR also has been coimmunoprecipitated from cells transfected with only HA-α2AR using anti-spinophilin 286–390 antibodies, indicating that it does not require the overexpression of Myc-spinophilin to detect an interaction with α2ARs (data not shown). Of particular interest, however, is the ability of the α2AR agonist, epineph-
treated with vehicle (2m) theraf-ras cascade, might disrupt pre-existing and significantly extend previous findings of in vitro loops (30, 40, 41). In some cases, the interactions are fostered by protein-coupled receptors via their C terminus (34–40) or 3i that these interactions are fostered by agonist binding to the region that shares little homology with what is otherwise a ordination of signal transduction events mediated by spinophilin, suggesting that regulated interactions with spi- ntagged spinophilin in an agonist-regulated fashion. A a proteins (44). The demonstrated to interact with 14-3-3z d arrests (42), whereas interactions appear to affect receptor trafficking (43). In some cases, interactions may be critical for receptor traffick- ing (45).

Interactions between α2ARs and spinophilin in the cell should be considered in the context of interactions between the 3i loop of α2ARs and other protein partners. Regions of the third intracellular loop of the α2AR have been shown to inter- act with 14-3-3z (30), β-arrestin (41) and heterotrimeric G proteins (44). The α2AR and α2AR 3i loops also have been shown to interact with 14-3-3z (30). These interactions with 14-3-3z are competed for by a phosphorylated peptide of raft that blocks raft-14-3-3 interactions (45), suggesting that receptor activation of downstream signaling pathways, such as the raf-ras cascade, might disrupt pre-existing α2AR-14-3-3 interactions, or vice versa. Interactions between α2AR and β-arrestin are expected to occur following agonist-evoked G protein-coupled receptor kinase-mediated α2AR phosphorylation (10). The 3i loop sequence employed for the α2AR in these studies includes regions that have been proposed to contribute to interactions with heterotrimeric G proteins (44), but these amphipathic helical sequences are not present in the amino acids encoded by the α2AR and α2AR 3i loop ligands. The ability of all three 3i loop ligands to interact with spinophilin comparably (e.g., Fig. 2) suggests that the α2AR-spinophilin interactions can occur independent of interactions with G proteins. It also is probable that α2AR-spinophilin interactions do not interact with G proteins, because agonist occupancy of the α2AR increases the amount of α2AR that can immunoprecipitate with spinophilin. Because agonist occupancy of α2AR also favors receptor interactions with G proteins (46), it is likely that the α2AR can interact simulta- neously with spinophilin and its cognate G protein. What remains to be established is whether this agonist-modulated interaction with spinophilin regulates acute or tonic receptor-mediated signaling, by analogy with findings for the D1 dopamine receptor (39, 47, 48) or mediates retention at the basolat- eral surface of polarized epithelial cells (Fig. 1), previously demonstrated to require the 3i loop of the AR subtypes α2A (21) and α2B (22).

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