0026-895X/05/6705-1690–1696$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 67:1690–1696, 2005

Vol. 67, No. 5
5215/1998165
Printed in U.S.A.

α2-Adrenergic Agonist Enrichment of Spinophilin at the Cell Surface Involves $\beta\gamma$ Subunits of G_i Proteins and Is Preferentially Induced by the α_{2A}-Subtype

Ashley E. Brady, Qin Wang, Patrick B. Allen, Mark Rizzo, Paul Greengard, and Lee E. Limbird

Departments of Pharmacology (A.E.B., Q.W., L.E.L.) and Molecular Physiology and Biophysics (M.R.), Vanderbilt University Medical Center, Nashville, Tennessee; Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, New York (P.G.); and Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut (P.B.A.)

Received July 26, 2004; accepted February 10, 2005

ABSTRACT

Agonist activation regulates reciprocal interactions of spinophilin and arrestin with the α_{2A}- and α_{2B}-adrenergic receptor (AR) subtypes via their 3i loop. Because arrestin association with G protein-coupled receptor is preceded by redistribution of arrestin to the cell surface, the present studies explored whether agonist activation of the α_{2A}- and α_{2B}-AR subtypes also led to spinophilin enrichment at the cell surface. Live cell imaging studies using a green fluorescent protein-tagged spinophilin examined spinophilin localization and its regulation by α_2-AR agonist. Agonist activation of α_{2A}-AR preferentially, compared with the α_{2B}-AR, led to spinophilin enrichment at the cell surface in human embryonic kidney 293 cells and in mouse embryo fibroblasts derived from spinophilin null mice. Activation of the ΔLEESSSSα_{2A}-AR, which has enriched association with spinophilin compared with the wild-type (WT) α_{2A}-AR, does not show an enhanced redistribution of spinophilin to the surface compared with WT α_{2A}-AR, demonstrating that the ability or affinity of the receptor in binding spinophilin may be independent of the ability of the receptor to effect spinophilin redistribution to the surface. Agonist-evoked enrichment of spinophilin at the cell surface seems to involve downstream signaling events, manifested both by the pertussis toxin sensitivity of the process and by the marked attenuation of spinophilin redistribution in cells expressing the β-adrenergic receptor kinase-C tail, which sequesters $\beta\gamma$ subunits of G proteins. Together, the data suggest that agonist-evoked spinophilin enrichment at the cell surface is caused by receptor-evoked signaling pathways and is independent of the affinity of the receptor for the spinophilin molecule.

The α_2-adrenergic receptors (ARs) are members of the large superfamily of G protein-coupled receptors. There are three α_2-AR subtypes (α_{2A}, α_{2B}, and α_{2C}), each of which is activated by the endogenous catecholamines epinephrine and norepinephrine and performs multiple physiological functions via pertussis toxin-sensitive G_i/G_o proteins (Limbird, 1988). Cellular signaling pathways regulated by the α_{2A}-AR subtype in native cells include inhibition of adenylyl cyclase, activation of receptor-operated K^+ channels, inhibition of voltage-gated Ca^{2+} channels, and activation of the mitogen-activated protein kinase cascade (Limbird, 1988; Kobilka, 1992; Richman and Regan, 1998).

Regions of the 3i loops of the α_{2A}- and α_{2B}-AR subtypes not implicated in G protein coupling have been demonstrated to be critical for stabilization of these subtypes at the basolateral surface of polarized renal epithelial cells in culture (Edwards and Limbird, 1999). A search for proteins localized at or near the cell surface that interact with the 3i loop of the α_2-AR and thus could be responsible for this stabilization resulted in the identification of the protein spinophilin (Richman et al., 2001). Spinophilin (Allen et al., 1997; Satoh et al., 1998) is a ubiquitously expressed, multidomain-containing

This work was supported by National Institutes of Health grant DK43879 (to L.E.L.). A.E.B. was funded by the Ruth L. Kirschstein National Service Award from the Neurogenomics Training grant MH65215. Experiments, data analysis, and presentation were performed on a Zeiss LSM510 Laser-Scanning Confocal Microscope through the use of the VUMC Cell Imaging Shared Resource (supported by National Institutes of Health grants CA68485, DK20593, DK58404, and HD15052). Cell culture media were prepared by the Diabetes Research and Training Center Cell Culture Core facility (supported by National Institutes of Health grant DK20593).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.005215.

ABBREVIATIONS: AR, adrenergic receptor; PP1, protein phosphatase 1; PDZ, postsynaptic density 95/disc-large/zona occludens; MDCK, Madin-Darby canine kidney; GFP, green fluorescent protein; HA, hemagglutinin; aa, amino acid(s); HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; MEF, mouse embryo fibroblast; GRK2, G protein receptor kinase 2; βARK, β-adrenergic receptor kinase; Sp, spinophilin; WT, wild-type; UK-14,304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine.

protein that possesses domains for F-actin binding, protein phosphatase 1 (PP1) binding, a single PDZ domain, and three coiled-coil domains. Spinophilin is endogenously enriched under the basolateral domain of cultured renal epithelial cells, Madin-Darby canine kidney (MDCKII) cells (Satoh et al., 1998; Richman et al., 2001). Brady et al. (2003) demonstrated that spinophilin does, in fact, contribute to stabilization of α_2-AR at the cell surface. In addition to its GPCR-interacting domain (Smith et al., 1999; Richman et al., 2001; Wang and Limbird, 2002), the other domains of spinophilin may allow for the formation of multiprotein complexes in intact cells that contribute to receptor localization and signaling complex formation.

Recent studies have shown that spinophilin and arrestin share regions of interaction in the 3i loop of α_{2A}-AR and α_{2B}-AR and that agonist occupancy of these receptors enhances spinophilin as well as arrestin association with the receptor (Wang and Limbird, 2002). Because agonist-induced association of arrestin with GPCR is preceded by agonist-enhanced translocation of arrestin to the cell surface, and because arrestin versus spinophilin interactions with either the α_{2A}-AR or α_{2B}-AR subtypes are reciprocal in nature (Wang et al., 2004), the present study examines whether α_2-AR activation enriches spinophilin localization at the cell surface, using spinophilin-GFP fusion proteins to monitor spinophilin localization in live cells over time.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) was prepared by the Cell Culture Core, a facility sponsored by the Diabetes Research and Training Center at Vanderbilt University Medical Center. Fetal calf serum was purchased from Atlanta Biologicals (Norcross, GA). The pEGFP-C1-spinophilin cDNA was a gift from Roger J. Colbran (Department of Molecular Physiology and Biophysics, Vanderbilt University). The retroviral vector pLEGFP-N1 was purchased from BD Biosciences Clontech (Palo Alto, CA). The retroviral vectors pBabe-HA-α_{2A}-AR and pBabe-HA-α_{2B}-AR were kindly provided by Drs. Dan Gil and John Donello (Allergan, Irvine, CA). Poly-D-lysine, polybrene, and puromycin were purchased from Sigma-Aldrich (St. Louis, MO), and pertussis toxin was from List Biological Laboratories Inc. (Campbell, CA). UK-14,304 was ordered from Sigma/RBI (Natick, MA). MatTek dishes were purchased from MatTek (Ashland, MA). Rat anti-HA 3F10 high affinity was from Roche Diagnostics (Indianapolis, IN). Spinophilin (aa 286–390) antibody was from Dr. Colbran (Department of Molecular Physiology and Biophysics, Vanderbilt University) and purified by us (Richman et al., 2001).

Cell Culture. HEK 293 cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37°C, 5% CO_2. Mouse embryo fibroblasts (MEFs) were isolated from spinophilin knockout ($Sp^{-/-}$) mice (Feng et al., 2000) as described previously (Brady et al., 2003). MEFs were immortalized via standard NIH3T3 protocol (Todaro and Green, 1963). Cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37°C, 5% CO_2.

Transfection or Transduction of Cells. HEK 293 cells were transfected using FuGENE 6 reagent (Roche Diagnostics) according to the manufacturer's direction with a pCMV4 vector backbone (control) or pCVM4-HA-α_{2A} AR. The pCMV4 vector for this receptor has been described previously (Schramm and Limbird, 1999). The cDNA encoding the C terminus of the G protein receptor kinase 2 (GRK2), known as the β-adrenergic receptor kinase (βARK)-C tail, was obtained from Marc Caron (Duke University, Durham, NC).

Immortalized $Sp^{-/-}$ MEFs were transduced with retroviral vectors encoding HA-tagged α_2-AR receptors and GFP-tagged spinophilin constructs as described previously (Brady et al., 2003). Transduced cells were selected for pBabe-α_2-AR expression by treatment for 36 h with 4 μg/ml puromycin (the pBabe retroviral vector carries the resistance gene for puromycin). Stable α_2-AR-expressing clones were isolated by standard ring-cloning methods and screened via radioligand binding analysis, using the radiolabeled α_2-AR-antagonist [^{3}H]rauwolscine, essentially as described previously (Edwards and Limbird, 1999). To assess the localization of spinophilin in live cells, either holo-spinophilin (Sp1-817) or spinophilin amino acids 151 to 444 were amplified via polymerase chain reaction extension from wild-type spinophilin using primers engineered to contain unique restriction sites HindIII and SalI. The amplified polymerase chain reaction product was then subcloned into the pLEGFP-N1 retroviral backbone at the HindIII and SalI sites. The final constructs were verified by sequencing analysis. MEFs stably expressing HA-α_{2A}- or HA-α_{2B}-AR were then transduced with the pLEGFP-spinophilin retroviral constructs and selected by growth in 500 μg/ml G418 (Geneticin).

Determination of Receptor Density. Permanent transformants of MEFs expressing either the HA-α_{2A}- or HA-α_{2B}-AR were assayed for functional receptor density using standard saturation binding protocols using [^{3}H]rauwolscine (HA-α_{2A}-AR) (PerkinElmer Life and Analytical Sciences, Boston, MA) or [^{3}H]RX 821002 radioligand (HA-α_{2B}-AR) (PerkinElmer Life and Analytical Sciences) (Edwards and Limbird, 1999) and nonlinear regression analysis using Prism (GraphPad Software Inc., San Diego, CA). The density of the MEFs expressing the HA-α_{2A}-AR was 2.0 pmol/mg membrane protein, and the density of the MEFs expressing the HA-α_{2B}-AR was 1.4 pmol/mg membrane protein.

Live Cell Imaging. HEK 293 or MEFs were plated the night before the assay on MatTek dishes (35 mm) coated with 2.5 $\mu g/cm^2$ poly-D-lysine at 3.5×10^5 cells per dish in medium containing the α_2-AR antagonist phentolamine (10^{-6} M) to eliminate effects of catecholamines that might be present in the serum-containing DMEM. The day of the assay, cells were washed 2×30 min in serum-free DMEM containing 0.01% bovine serum albumin and 3×30 min in serum-free DMEM supplemented with 20 mM HEPES. All washes were performed at 37°C. Quantitative, live cell confocal microscopy was performed using a Zeiss LSM510 confocal microscope equipped with a 488-nm argon/krypton laser and fitted with a heated and humidified chamber system. All experiments were performed at 37°C using a 40×/1.3 numerical aperture oil immersion lens. Emitted fluorescence was detected with a 550-nm-long pass filter.

Data capture was carried out in the following manner. First, a z-stack of the cell of interest was acquired before drug treatment, and then the time series was begun, where sequential images at a single, central plane were captured at 1-min intervals for the duration of the time course. The indicated ligand was introduced immediately after the first scan. After the completion of the time course, usually 10 min in the presence of UK-14,304, a post-drug z-stack was acquired (specific incubation conditions are given in the figure legends). GFP-spinophilin redistribution after drug treatment was reflected by an increase in plasma membrane fluorescence, quantified as changes in pixel intensity. Using MetaMorph software (Universal Imaging Corporation, Downingtown, PA), measurements were made by selecting a region encompassing the entire plasma membrane (defined as total), and then selecting a region just inside the plasma membrane (defined as inside). The difference between these two measurements reflects the defined membrane area. The product of the number of pixels and the average pixel intensity was calculated for the total area as well as for the defined membrane area for each cell both before ($t = 0$) and after ($t = 10$ min) drug treatment. The membrane pixel intensity was then expressed as a fraction of total pixel intensity. Changes in fluorescence over time are expressed as percentage of time 0.

Coimmunoisolation of HA-α_2AR with Spinophilin. Coimmunoisolation of HA-α_2AR subtypes with spinophilin was performed as described previously (Wang and Limbird, 2002) with a few modifications. MEFs transduced with HA-α_2AR subtypes were stimulated with or without agonist (100 μM epinephrine + 1 μM propranolol) for 5 min. Endogenous spinophilin was detected with spinophilin antibodies in immunocomplexes isolated by rat anti-HA antibodies.

Results

Enrichment of GFP-Spinophilin at the Plasma Membrane of HEK 293 Cells Mediated by α_{2A}-AR. Previous findings from our laboratory have demonstrated that all three α_2-AR subtypes (α_{2A}, α_{2B}, and α_{2C}) interact with the multidomain protein spinophilin via their third intracellular loop (Richman et al., 2001). Interactions of the α_{2A}-AR and α_{2B}-AR subtypes with spinophilin are enhanced by agonist, as revealed by the enrichment of spinophilin in α_2-AR-containing immunoisolates after treatment of target cells with an α_2-AR agonist (Richman et al., 2001; Wang and Limbird, 2002). This led us to the hypothesis that the increase in α_2-AR-associated spinophilin detected in coimmunoisolation assays might be due, at least in part, to an agonist-induced enrichment of spinophilin at the plasma membrane, by analogy with agonist-induced redistribution of another 3i loop-interacting protein to the cell surface, arrestin (Barak et al., 1997; Groarke et al., 1999). Although we did observe some increase in the membrane localization of *endogenous* spinophilin in both MEFs and SCG neurons (data not shown), the apparent magnitude of the change was not equivalent in every cell, and by using antibody staining, we could not

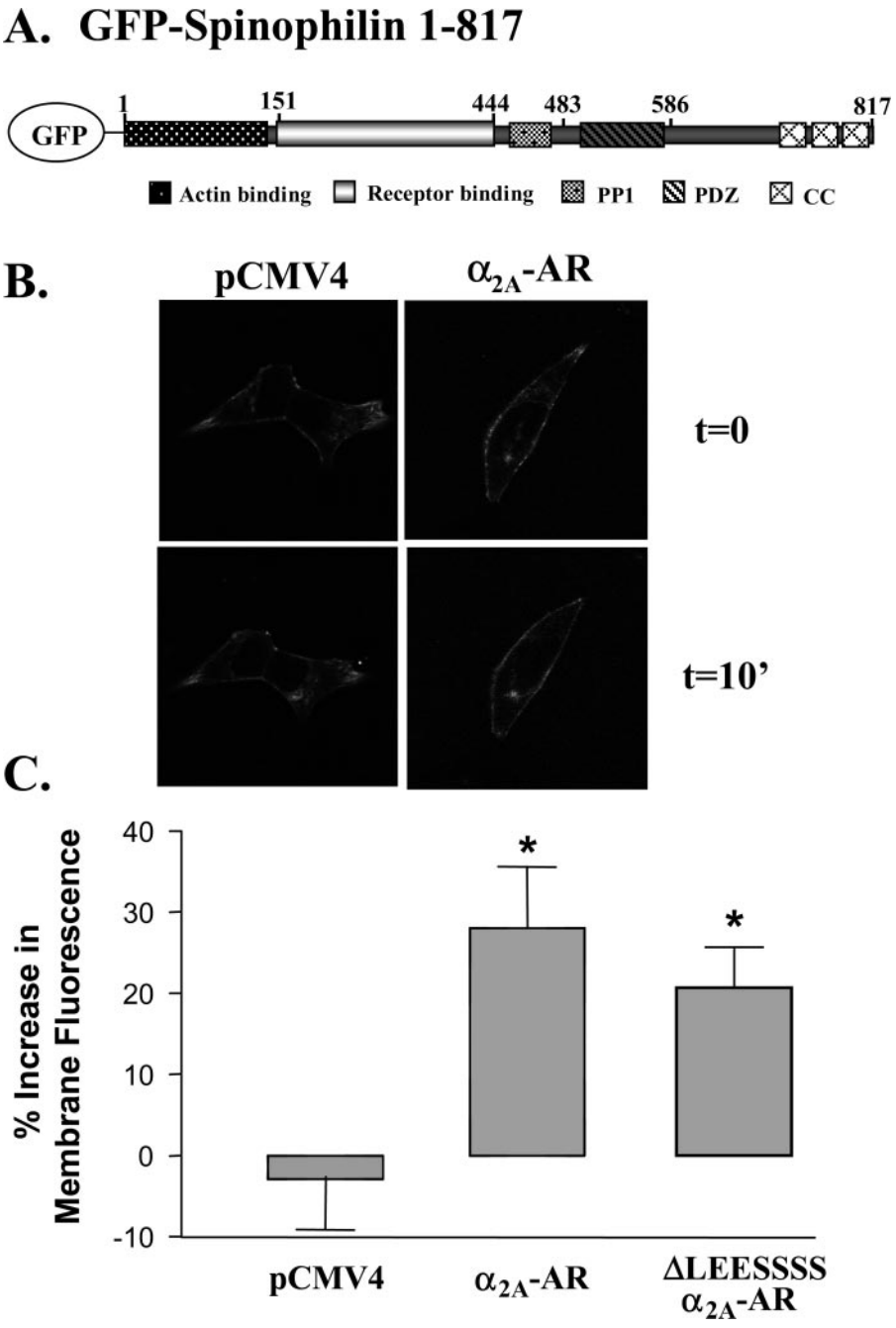

Fig. 1. Agonist-elicited enrichment of spinophilin at the cell surface in α_{2A}-AR-expressing cells. A, schematic diagram of the N-terminally fused GFP-spinophilin protein. Spinophilin is a multidomain protein composed of an actin binding (aa 1–151), GPCR binding (aa 151–444), PP1 binding, and regulatory (aa 444–485), PDZ (aa 483–586), and C-terminal coiled-coil domains. B, confocal images of HEK 293 cells transiently expressing the α_{2A}-AR or exposed to the same manipulation but transfected with a pCMV4 empty backbone vector. Images shown here were captured at time 0 and 10 min after agonist treatment with 10 μM UK-14,304, an α_2-adrenergic agonist, as described under *Materials and Methods*. C, quantitative analysis of confocal images, performed as detailed under *Materials and Methods*, reveals an approximately 30 and 22% increase in membrane-associated spinophilin after agonist exposure in cells expressing α_{2A}-AR and ΔLEESSSSα_{2A}-AR, respectively. Data are the means ± S.E.M. from 11 measurements in two independent experiments (pCMV4) and 19 measurements in three independent experiments (α_{2A}-AR), and means ± range from two measurements in two independent experiments (ΔLEESSSSα_{2A}-AR).

analyze the same cell before and after drug treatment, because we had to fix and permeabilize the cell preparations to identify endogenous spinophilin. Thus, to evaluate agonist-evoked spinophilin redistribution in a more quantitative manner within single cells (using each cell as its own control), a cDNA encoding a GFP-spinophilin fusion protein was coexpressed with α_{2A}-AR in HEK 293 cells, and the localization of GFP-spinophilin was monitored in real-time by confocal microscopy after stimulation of the HEK 293 cells with the α_2-AR agonist UK-14,304. Figure 1A provides a schematic diagram of N-terminal fusion of GFP with the multidomain protein spinophilin.

Unlike for arrestin, which is principally cytosolic (Barak et al., 1997), a population of spinophilin seems to constitutively associate at or just underneath the surface membrane as well as in a cytosolic pool (Satoh et al., 1998; Richman et al., 2001). Our data indicate that, under basal conditions, 28.2 ± 1.5 and 28.3 ± 2.6% of the total cellular GFP-spinophilin is localized to the cell membrane in HEK 293 cells transfected with pCMV4 alone or pCMV4 encoding the α_{2A}-AR, respectively. The preexistence of a considerable fraction of spinophilin at the cell surface masks the quantitative extent of spinophilin redistribution using visual inspection alone (Fig. 1B), but quantitative confocal microscopy analysis (see *Materials and Methods*) reveals a 30% increase in GFP-spinophilin at the cell surface after agonist activation of the α_{2A}-AR (Fig. 1C). No detectable increase is observed in cells expressing the control vector pCMV4. This redistribution of GFP-spinophilin to the plasma membrane was detected as early as 2 min after the addition of agonist and did not seem to increase further after 10 min; nor did we observe a reversal of the association with longer time points (i.e., 30 min; data not shown). It is interesting that activation of the ΔLEESSSSα_{2A}-AR, which has enriched association with spinophilin compared with the WT α_{2A}-AR when examined in immunoisolation experiments (Wang and Limbird, 2002), does not show an enhanced redistribution of spinophilin to the surface compared with WT α_{2A}-AR (Fig. 1C), and—if anything—is slightly attenuated in its effectiveness in effecting spinophilin redistribution. These data suggest the possibility that receptor-spinophilin complex formation may be an event independent of agonist-induced spinophilin enrichment at the cell surface.

It was of interest to us in our preliminary studies that a similar redistribution of spinophilin was not seen after agonist activation of the α_{2B}-AR expressed in HEK 293 cells. However, because we were not able to achieve similar expression of the α_{2B}-AR subtype as the α_{2A}-AR subtype after transient transfection in these cells, we were concerned that receptor density, per se, might be responsible for this apparent subtype-selective response, and we chose to pursue possible subtype selectivity of this response in permanent, clonal cell lines.

Agonist-Mediated Redistribution of Spinophilin-GFP in $Sp^{-/-}$ MEFs. We chose to explore the possible subtype selectivity of α_2-AR-mediated enrichment of spinophilin at the cell surface in MEFs derived from mice null for spinophilin ($Sp^{-/-}$) expressing either the α_{2A}-AR (2.0 pmol/mg protein) or the α_{2B}-AR (1.4 pmol/mg protein) in clonal cell lines selected after retroviral transduction with either receptor subtype. Selection of the $Sp^{-/-}$ genetic background to explore real-time distribution of spinophilin-GFP was intended to increase the sensitivity of our signal.

Agonist activation of the α_{2A}-AR subtype in MEFs evokes a

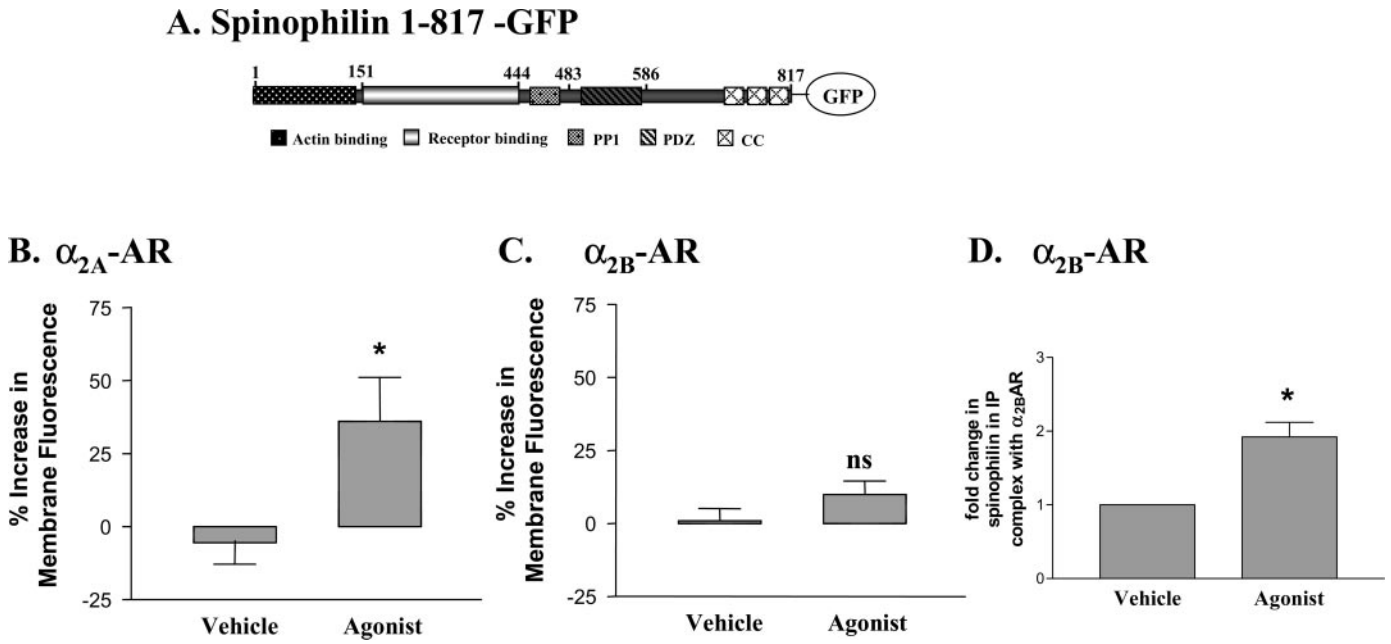

Fig. 2. Enrichment of spinophilin at the cell surface is selective for the α_{2A}-AR subtype, as revealed in permanent transformants of $Sp^{-/-}$ mouse embryo fibroblasts. A, schematic diagram of the spinophilin-GFP fusion protein introduced into $Sp^{-/-}$ MEFs permanently expressing α_{2A}-AR (B) or α_{2B}-AR (C), as described under *Materials and Methods*. B and C, membrane fluorescence changes in MEFs expressing α_{2A}-AR (B) or α_{2B}-AR (C). Data are the mean ± S.E.M. from five measurements in two independent experiments (vehicle) and nine measurements in five independent agonist-treated experiments (UK-14,304) for α_{2A}-AR-expressing cells (B) and 21 measurements in three independent experiments (vehicle) and 22 measurements in four independent agonist-treated experiments (UK-14,304) for α_{2B}-AR-expressing cells (C). D, agonist-enhanced α_{2B}-AR association with spinophilin in MEFs. *, $p < 0.05$, comparing the fold change in spinophilin association with agonist added, compared with no agonist added (the latter defined as control, or 1.0-fold), $n = 3$. The agonist used in the coimmunoprecipitation studies was 100 μM epinephrine + 1 μM propranolol.

statistically significant increase in cell surface-associated spinophilin-GFP (Fig. 2B). Statistically significant increase in cell surface-associated spinophilin-GFP, however, is not observed after agonist activation of the α_{2B}-AR subtype in this same cellular background (Fig. 2C), even though agonist activation increases the association of α_{2B}-AR with spinophilin as detected in coimmunoisolation assays (Fig. 2D), a finding that shows that agonist occupancy of the α_{2B}-AR can elicit its characteristic association with spinophilin. These data suggest that, even in cells expressing the α_{2B}-AR at densities comparable with those of the α_{2A}-AR, the redistribution of spinophilin is more readily detectable in cells expressing the α_{2A}-AR subtype and that redistribution of spinophilin to the surface may be independent of the affinity of the receptor subtype for spinophilin, which is further revealed by studies with solely the receptor binding domain of spinophilin (see below).

For the studies in MEFs, a C-terminal GFP fusion with spinophilin was used (Fig. 2A), simply because the construction of the retroviral vector for transduction of these cells was more easily achieved in this configuration (Fig. 2A). The comparable findings using either N- or C-terminal GFP-spinophilin fusion proteins (cf. Fig. 1C with Fig. 2B) affirm that the present observations are not attributable to steric properties defined by the locus of the GFP fusion.

The Receptor Binding Domain of Spinophilin Is Not Sufficient for Agonist-Induced Enrichment at the Plasma Membrane. As a means to ascertain whether the receptor binding domain of spinophilin is sufficient for agonist-induced redistribution of spinophilin to the cell surface, or whether other domains are necessary, we expressed just the receptor binding domain of spinophilin (aa 151–444) (Fig. 3A) as a GFP fusion protein. In contrast to agonist-evoked enrichment of holo-spinophilin-GFP at the cell surface, no such enrichment occurred after UK-14,304 treatment of cells expressing Sp151-444-GFP (Fig. 3B). These findings suggest that receptor-spinophilin protein-protein interactions per se are not sufficient to permit the agonist-stimulated enrichment of spinophilin at the cell surface, implying that other domains of spinophilin, and thus other mechanisms, are involved, including the participation of downstream signaling pathways.

Agonist-Mediated Redistribution of GFP-Spinophilin to the Plasma Membrane Requires $G_{\beta\gamma}$. We were curious whether α_{2A}-AR interaction with its cognate G protein G_i/G_o was critical for enrichment of spinophilin at the cell surface after agonist activation. To test this hypothesis, MEFs were incubated with 100 ng/ml pertussis toxin overnight to ADP-ribosylate MEF G_i and thus disrupt the ability of the α_{2A}-AR to interact with the G protein. As can be seen in Fig. 4A, pertussis toxin treatment diminished the extent of α_{2A}-AR-mediated spinophilin redistribution to the cell surface after UK-14,304 treatment of either HEK 293 cells or MEFs. Parallel studies demonstrate that the G protein-dependent mitogen-activated protein kinase signaling activated by the α_{2A}-AR in HEKs and MEFs also is attenuated by pertussis toxin treatment, confirming that toxin exposure of the cells was successful in modifying at least a fraction of the G_i- and perturbing G_i-dependent functions. These findings suggest either that receptor-G protein interactions per se or receptor-evoked signaling pathways contribute to the enrichment of spinophilin at the cell surface after agonist activation of the receptor.

To further address the possible role of G proteins in α_{2A}-AR-evoked spinophilin enrichment at the cell surface, we overexpressed the C-terminal domain of βARK1 (or GRK2) to sequester the βγ subunits of G proteins (Koch et al., 1994). We chose this approach because we have recently observed that spinophilin association with the α_{2A}-AR seems to recognize a complex containing the receptor and the βγ subunits of heterotrimeric G proteins, by analogy with the basis for GRK association with the agonist-activated GPCRs (Wang et al., 2004). The findings in Fig. 4B suggest that spinophilin enrichment at the cell surface after agonist treatment of cells also involves $G_{\beta\gamma}$ subunits, because expression of the βγ-interacting domain of β-adrenergic receptor kinase, its C terminus (βARK-C tail), disrupts the ability to detect agonist-evoked spinophilin enrichment at the cell surface in HEK 293 cells. These data, then, emphasize the importance of G protein activation in α_{2A}-AR-mediated enrichment of spinophilin at the cell surface.

Discussion

The present study demonstrates three important findings. First, agonist activation of the α_{2A}-AR enriches spinophilin association with the plasma membrane, a finding that has not been reported previously for GPCRs that interact with spinophilin. Second, redistribution to the cell

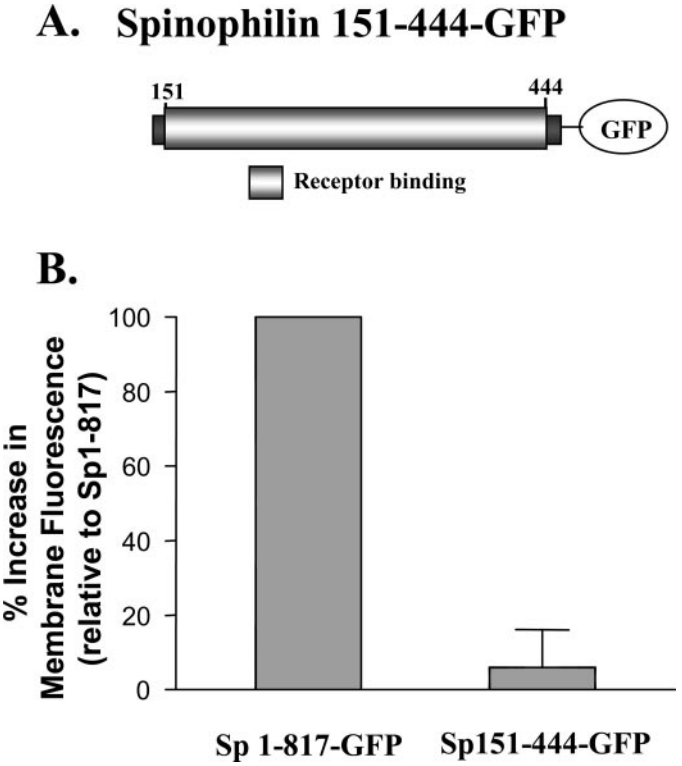

Fig. 3. α_2-AR-interacting domain of spinophilin is not sufficient to mediate agonist-elicited enrichment of spinophilin at the cell surface. The GPCR-interacting domain of spinophilin, Sp151-444, was fused to GFP at the C terminus of this domain and transduced into $Sp^{-/-}$ MEFs permanently expressing the α_{2A}-AR (A). The data are expressed as percentage of increase in membrane fluorescence, relative to the increase observed with holo-spinophilin-GFP (Sp1-817) (B). The data shown are the means ± S.E.M. of 21 independent measurements in three separate experiments.

surface involves the $\beta\gamma$ subunits of the pertussis toxin-sensitive G protein G_i. This finding means either that receptor-elicited signaling via $\beta\gamma$-mediated pathways is involved in spinophilin enrichment at the cell surface or that a receptor-$\beta\gamma$ complex, enriched by agonist activation of the receptor, serves as the "docking site" for spinophilin, or both. Third, the ability of α_2-AR subtypes to associate with spinophilin is independent of agonist-evoked spinophilin enrichment at the cell surface. Several lines of evidence are consistent with this interpretation. First, the observation that the receptor-interacting domain of spinophilin, Sp151-444, is insufficient to respond to agonist activation of the α_{2A}-AR for redistribution to the surface (Fig. 3) is consistent with α_{2A}-AR downstream signaling

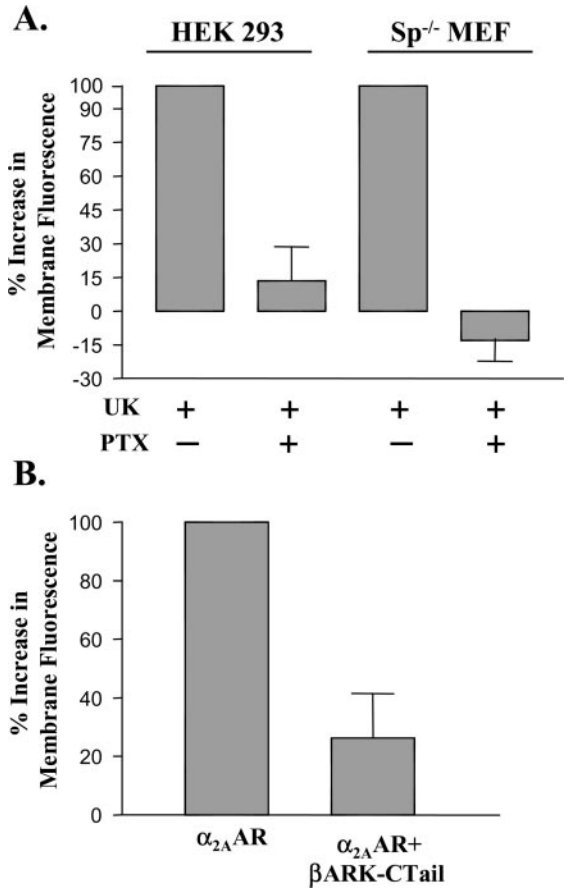

Fig. 4. $\beta\gamma$ subunits of pertussis toxin-sensitive G proteins are implicated in α_{2A}-AR-elicited enrichment of spinophilin at the cell surface. A, HEK 293 cells or $Sp^{-/-}$ MEFs expressing α_{2A}-AR transiently (HEK 293) or permanently ($Sp^{-/-}$ MEFs) were treated with 10 μM UK-14,304 under control conditions or after exposure overnight to 100 ng/ml pertussis toxin, as described under *Materials and Methods*. The data shown are the mean ± S.E.M. from 14 separate determinations in three independent experiments (HEK 293) and 16 separate determinations in three independent experiments ($Sp^{-/-}$ MEF) and are expressed as percentage of "control", defined as the increase measured in HEK 293 cells or in $Sp^{-/-}$ MEFs expressing α_{2A}-AR in the absence of pertussis toxin, after 10 min stimulation with 10 μM UK-14,304 versus vehicle alone. B, HEK 293 cells were transfected with a cDNA encoding the α_{2A}-AR with or without a cDNA encoding the βARK-C terminus (βARK-C tail). Control cells (α_{2A}-AR) were cotransfected with a pCMV4 backbone cDNA. The data are shown as percentage of increase in membrane fluorescence compared with control conditions, defined as the increase measured after 10-min exposure to 10 μM UK-14,304 in HEK cells expressing α_{2A}-AR alone versus pCMV4. The data are the mean ± S.E.M. from 41 individual measurements in three separate experiments.

events contributing to agonist-enrichment of spinophilin at the plasma membrane, perhaps events involving phosphorylation and/or dephosphorylation of the other domains of spinophilin, including the actin binding, PP1 regulatory, PDZ, or coiled-coil domains. Second, stimulation of an α_{2A}-AR mutant lacking the GRK2 phosphorylation consensus sequence does not result in a greater enrichment of spinophilin at the cell surface than for WT α_{2A}-AR (Fig. 1C), despite the fact that this mutated receptor displayed an increased association with spinophilin in coimmunoisolation studies compared with WT α_{2A}-AR (Wang and Limbird 2002). Third, activation of the α_{2B}-AR does not lead to detectable redistribution of spinophilin (Fig. 2C), in contrast to findings for α_{2A}-AR, although the α_{2B}-AR readily complexes with spinophilin in MEFs (cf. Figure 2D) and in other cells. It is possible, of course, that both the α_{2A}-AR and α_{2B}-AR can effect spinophilin redistribution to the membrane, but the sensitivity of our measurements preclude detection of statistically significant redistribution in response to agonist activation of the α_{2B}-AR. Nonetheless, these findings indicate that there is a preferential capability of the α_{2A}-AR to effect redistribution of spinophilin to the cell surface. Because both α_{2A}-AR and α_{2B}-AR seem to couple to the similar G proteins and effector molecules, the apparently differential capacities of these subtypes to recruit spinophilin to the plasma membrane may be a mechanism contributing to subtype signaling diversity. For example, in dendritic spines of neurons, where spinophilin is enriched (Allen et al., 1997), the α_{2A}-AR subtype may be able to engage or amplify signaling pathways using spinophilin-associated proteins in a way that the α_{2B}-AR cannot.

Future studies will reveal the generality of this finding for other GPCRs, especially for those that have already been shown to interact with spinophilin (Smith et al., 1999), and their functional relevance in vitro and in vivo.

Acknowledgments

We thank Carol Ann Bonner and Sean B. Schaffer for excellent and dedicated technical support, and Drs. David Piston and Sam Wells (Department of Molecular Physiology and Biophysics, Vanderbilt University) for considerable technical advice.

References

Allen PB, Ouimet CC, and Greengard P (1997) Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proc Natl Acad Sci USA* **94:**9956–9961.

Barak LS, Ferguson SS, Zhang J, and Caron MG (1997) A β-arrestin/green fluorescent protein biosensor for detecting g protein-coupled receptor activation. *J Biol Chem* **272:**27497–27500.

Brady AE, Wang Q, Colbran RJ, Allen PB, Greengard P, and Limbird LE (2003) Spinophilin stabilizes cell surface expression of α2b-adrenergic receptors. *J Biol Chem* **278:**32405–32412.

Edwards SW and Limbird LE (1999) Role for the third intracellular loop in cell surface stabilization of the α2a-adrenergic receptor. *J Biol Chem* **274:**16331–16336.

Feng J, Yan Z, Ferreira A, Tomizawa K, Liauw JA, Zhuo M, Allen PB, Ouimet CC, and Greengard P (2000) Spinophilin regulates the formation and function of dendritic spines. *Proc Natl Acad Sci USA* **97:**9287–9292.

Groarke DA, Wilson S, Krasel C, and Milligan G (1999) Visualization of agonist-induced association and trafficking of green fluorescent protein-tagged forms of both β-arrestin-1 and the thyrotropin-releasing hormone receptor-1. *J Biol Chem* **274:**23263–23269.

Kobilka B (1992) Adrenergic receptors as models for g protein-coupled receptors. *Annu Rev Neurosci* **15:**87–114.

Koch WJ, Hawes BE, Inglese J, Luttrell LM, and Lefkowitz RJ (1994) Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G$\beta\gamma$-mediated signaling. *J Biol Chem* **269:**6193–6197.

Limbird LE (1988) Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB J* **2:**2686–2695.

Richman JG, Brady AE, Wang Q, Hensel JL, Colbran RJ, and Limbird LE (2001) Agonist-regulated interaction between α2-adrenergic receptors and spinophilin. *J Biol Chem* **276:**15003–15008.
Richman JG and Regan JW (1998) α2-Adrenergic receptors increase cell migration and decrease f-actin labeling in rat aortic smooth muscle cells. *Am J Physiol* **274:**C654–C662.
Satoh A, Nakanishi H, Obaishi H, Wada M, Takahashi K, Satoh K, Hirao K, Nishioka H, Hata Y, Mizoguchi A, et al. (1998) Neurabin-II/spinophilin. An actin filament-binding protein with one PDZ domain localized at cadherin-based cell-cell adhesion sites. *J Biol Chem* **273:**3470–3475.
Schramm NL and Limbird LE (1999) Stimulation of mitogen-activated protein kinase by G protein-coupled α_2-adrenergic receptors does not require agonist-elicited endocytosis. *J Biol Chem* **274:**24935–24940.
Smith FD, Oxford GS, and Milgram SL (1999) Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *J Biol Chem* **274:**19894–19900.
Todaro GJ and Green H (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol* **17:**299–313.
Wang Q and Limbird LE (2002) Regulated Interactions of the α2a adrenergic receptor with spinophilin, 14-3-3ζ and arrestin 3. *J Biol Chem* **277:**50589–50596.
Wang Q, Zhao J, Brady AE, Feng J, Allen PB, Lefkowitz RJ, Greengard P, and Limbird LE (2004) Spinophilin blocks arrestin actions in vitro and in vivo at G protein-coupled receptors. *Science (Wash DC)* **304:**1940–1944.

Address correspondence to: Dr. Qin Wang, Department of Pharmacology, Vanderbilt University Medical Center, 464 Robinson Research Bldg., Nashville, TN 37232-6600. E-mail: qin.wang@vanderbilt.edu