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Alpha2-adrenergic agonist enrichment of spinophilin at the cell surface involves βγ subunits of G_i proteins and is preferentially induced by the α_{2A}-subtype [¶¥]

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Running Title: α_2-AR-Regulated Membrane Enrichment of Spinophilin

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Abbreviations: 3i loop, third intracellular loop; α_2-AR, α_2-adrenergic receptor; BSA, bovine serum albumin; DPBS/CM, Dulbecco's phosphate buffered saline supplemented with 1mM $MgCl_2$ and 0.5mM $CaCl_2$; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; GPCR, G Protein-coupled receptor; HA, hemagglutinin; MEFs, mouse embryo fibroblasts; [PDZ, *P*SD-95, *D*iscs large, ZO-1]; PP1, protein phosphatase 1.

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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. Since arrestin association with GPCR 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 (GFP)-tagged spinophilin examined spinophilin localization, and its regulation by α_2-AR agonist. Agonist activation of α_{2A}-AR preferentially, when compared to the α_{2B}-AR, led to spinophilin enrichment at the cell surface in HEK293 cells and in mouse embryo fibroblasts derived from spinophilin null mice. Activation of the ΔLEESSSSα_{2A}-AR, which has enriched association with spinophilin compared to the WT α_{2A}-AR, does not show an enhanced redistribution of spinophilin to the surface when compared to 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 appears to involve downstream signaling events, manifest both by the pertussis toxin sensitivity of the process and by the marked attenuation of spinophilin redistribution in cells expressing the βARK-C tail, which sequesters $\beta\gamma$ subunits of G proteins. Taken together, the data suggest that agonist-evoked spinophilin enrichment at the cell surface is due to receptor-evoked signaling pathways and is independent of the affinity of the receptor for the spinophilin molecule.

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INTRODUCTION

The α_2-adrenergic receptors (α_2-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 (MAPK) cascade (Kobilka, 1992;Limbird, 1988;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, multi-domain-containing protein which 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.* demonstrated that spinophilin does, in fact, contribute to stabilization of α_2-AR at the cell surface (Brady *et al.*, 2003). 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

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allow for the formation of multi-protein 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). Since agonist-induced association of arrestin with GPCR is preceded by agonist-enhanced translocation of arrestin to the cell surface, and since 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.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (Gibco) 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. The pEGFP-C1-Spinophilin cDNA was a gift from Roger J. Colbran (Dept. of Molecular Physiology and Biophysics, Vanderbilt University). The retroviral vector pLEGFP-N1 was purchased from Clontech. 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, and Pertussis Toxin was from List Biological Laboratories Inc. UK 14,304 was ordered from Research Biochemical International. MatTek dishes were purchased from MatTek Corporation (Ashland, MA). Rat anti-HA 3F10 high affinity was from Roche. spinophilin (aa 286 – 390) antibody was from Dr. Colbran (Dept. 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% FCS, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37ºC/5% CO_2. Mouse Embryo Fibroblasts (MEFs) were isolated from spinophilin knock-out ($Sp^{-/-}$) 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% FCS, 2mM glutamine, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37ºC/5% CO_2.

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Transfection or Transduction of Cells

HEK 293 cells were transfected using FuGENE 6 reagent (Roche Molecular Biochemicals) 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 βARK-Ctail, was obtained from Marc Caron (Duke University).

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 hours with 4μg/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 151-444 were amplified *via* PCR extension from wild type spinophilin using primers engineered to contain unique restriction sites *Hind*III and *Sal*I. The amplified PCR product was then subcloned into the pLEGFP-N1 retroviral backbone at the *Hind*III and *Sal*I 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 500μg/mL G418.

Determination of Receptor Density

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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) (NEN) or [^{3}H]-RX 821002 radioligand (HA-α_{2B}-AR) (NEN)(Edwards and Limbird, 1999)and non-linear regression analysis using Graph-Pad prism. 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

HEK293 or MEFs were plated the night before the assay on Mat TEK dishes (35 mm) coated with 2.5 μg/cm^2 Poly-D-Lysine at 3.5 X 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 X 30 min in serum free (SF) DMEM containing 0.01% bovine serum albumen (BSA), and 3 X 30 min in SF DMEM supplemented with 20mM 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 40X N.A. 1.3 oil-immersion lens. Emitted fluorescence was detected with a 550 nm long pass filter.

Data capture was carried out in the following fashion. First, a z-stack of the cell of interest was acquired prior to drug treatment, then the time series was begun, where sequential images at a single, central plane were captured at 1 minute 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

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redistribution following drug treatment was reflected by an increase in plasma membrane fluorescence, quantified as changes in pixel intensity. Using Metamorph Software (Universal Imaging Corporation), 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 prior to (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 % of time 0.

Co-immunoisolation of HA-α_2AR with spinophilin.

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

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RESULTS

Enrichment of GFP-spinophilin at the plasma membrane of HEK293 cells mediated by α_{2A}-AR

Previous findings from our laboratory have demonstrated that all three α_2-adrenergic receptor (α_2-AR) subtypes (α_{2A}, α_{2B}, α_{2C}) interact with the multi-domain 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 following 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 co-immunoisolation 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 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 fashion within single cells (using each cell as its own control), a cDNA encoding a GFP-spinophilin fusion protein was co-expressed with α_{2A}-AR in HEK293 cells, and the localization of GFP-spinophilin was monitored in real-time by confocal microscopy following 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 multi-domain protein, spinophilin.

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Unlike for arrestin, which is principally cytosolic (Barak *et al.*, 1997), a population of spinophilin appears 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 HEK293 cells transfected with pCMV4 alone or pCMV4 encoding the α_{2A}-AR, respectively. The pre-existence 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 (Experimental Procedures) reveals a 30% increase in GFP-spinophilin at the cell surface following 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 minutes after the addition of agonist and did not appear 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). Interestingly, activation of the ΔLEESSSSα_{2A}-AR, which has enriched association with spinophilin compared to 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 when compared to 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 complexation 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 following 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

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α_{2A}-AR subtype following transient transfection in these cells, we were concerned that receptor density, *per se*, might be responsible for this apparent subtype-selective response, and 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 statistically significant increase in cell-surface associated spinophilin-GFP (Fig. 2B). Statistically significant increased in cell-surface associated spinophilin-GFP, however, are not observed following 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 co-immunoisolation assays (Fig. 2D), a finding which 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 to 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, below.

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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 vs. 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 if 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 (aa151-444) (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 following UK 14,304 treatment of cells expressing Sp151-444-GFP. 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 following agonist activation. To test this hypothesis, MEFs were incubated with 100 ng/ml pertussis toxin overnight in order 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 (PTx) diminished the extent of α_{2A}-AR-mediated spinophilin redistribution to the cell surface following UK 14,304 treatment of either

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HEK293 cells or MEFs. Parallel studies demonstrate that the G-protein dependent MAPK 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 β-adrenergic receptor kinase 1 (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 appears 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 following agonist treatment of cells also involves $G_{\beta\gamma}$ subunits, since 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 HEK293 cells. These data, then, emphasize the importance of G protein activation in α_{2A}-AR-mediated enrichment of spinophilin at the cell surface.

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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 GPCR that interact with spinophilin. Second, redistribution to the cell 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_2AR 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 is consistent with α_{2A}-AR downstream signaling 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 co-immunoisolation studies compared to 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* Fig. 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

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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. Since both α_{2A}-AR and α_{2B}-AR appear 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 signalling 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 signalling pathways utilizing 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*.

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FOOTNOTES

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FIGURE LEGENDS

Figure 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 (aa1-151), GPCR-binding (aa151-444), PP1-binding and regulatory (aa444-485), PDZ (aa483-586) and C-terminal coiled-coil domains. **B.** Confocal images of HEK293 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 minutes following agonist treatment with 10 μM UK14,304, an α_2-adrenergic agonist, as described in Experimental Procedures. **C.** Quantitative analysis of confocal images, performed as detailed in Experimental Procedures, reveals an approximately 30% and 22% increase in membrane-associated spinophilin following agonist exposure in cells expressing α_{2A}-AR and ΔLEESSSSα_{2A}-AR, respectively. Data are the means ± SEM from 11 measurements in 2 independent experiments (pCMV4) and 19 measurements in 3 independent experiments (α_{2A}-AR), and means ± range from 2 measurements in 2 independent experiments (ΔLEESSSSα_{2A}-AR).

Figure 2. The 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 (panel B) or α_{2B}-AR (panel C), as described in Experimental Procedures. **B.** & **C.** Membrane fluorescence changes in MEFs expressing α_{2A}-AR (panel B) or α_{2B}-AR (panel C). Data are the mean ± SEM from 5 measurements in 2 independent

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experiments (vehicle) and 9 measurements in 5 independent agonist-treated experiments (UK 14,304) for α_{2A}-AR-expressing cells (panel B) and 21 measurements in 3 independent experiments (vehicle) and 22 measurements in 4 independent agonist-treated experiments (UK 14,304) for α_{2B}-AR-expressing cells (panel 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 co-IP studies was 100μM epinephrine + 1 μM propranolol (Wang *et al.*, 2002).

Figure 3. The 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. The data are expressed as % increase in membrane fluorescence, relative to the increase observed with holo-spinophilin-GFP (Sp1-817). The data shown are the means ± SEM of 21 independent measurements in 3 separate experiments.

Figure 4. The βγ subunits of pertussis toxin-sensitive G proteins are implicated in α_{2A}-AR-elicited enrichment of spinophilin at the cell surface. A. HEK293 cells or $Sp^{-/-}$ MEFs expressing α_{2A}-AR transiently (HEK 293) or permanently ($Sp^{-/-}$ MEFs) were treated with 10 μM UK14,304 under control conditions, or following exposure overnight to 100 ng/mL pertussis toxin, as described in Experimental Procedures. The data shown are the mean ± SEM from 14 separate determinations in 3 independent experiments (HEK293), and 16 separate determinations

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in 3 independent experiments ($Sp^{-/-}$ MEF), and are expressed as % of "control," defined as the increase measured in HEK293 cells or in $Sp^{-/-}$ MEFs expressing α_{2A}-AR in the absence of pertussis toxin, following 10 min stimulation with 10 μM UK14,304 *vs.* vehicle alone. **B.** HEK293 cells were transfected with a cDNA encoding the α_{2A}-AR with or without a cDNA encoding the βARK-C terminus (βARK-CTail). Control cells (α_{2A}-AR) were cotransfected with a pCMV4 backbone cDNA. The data are shown as % increase in membrane fluorescence compared to control conditions, defined as the increase measured following 10 min exposure to 10 μM UK14,304 in HEK cells expressing α_{2A}-AR alone *vs.* pCMV4. The data are the mean ± SEM from 41 individual measurements in 3 separate experiments.

Fig 1.

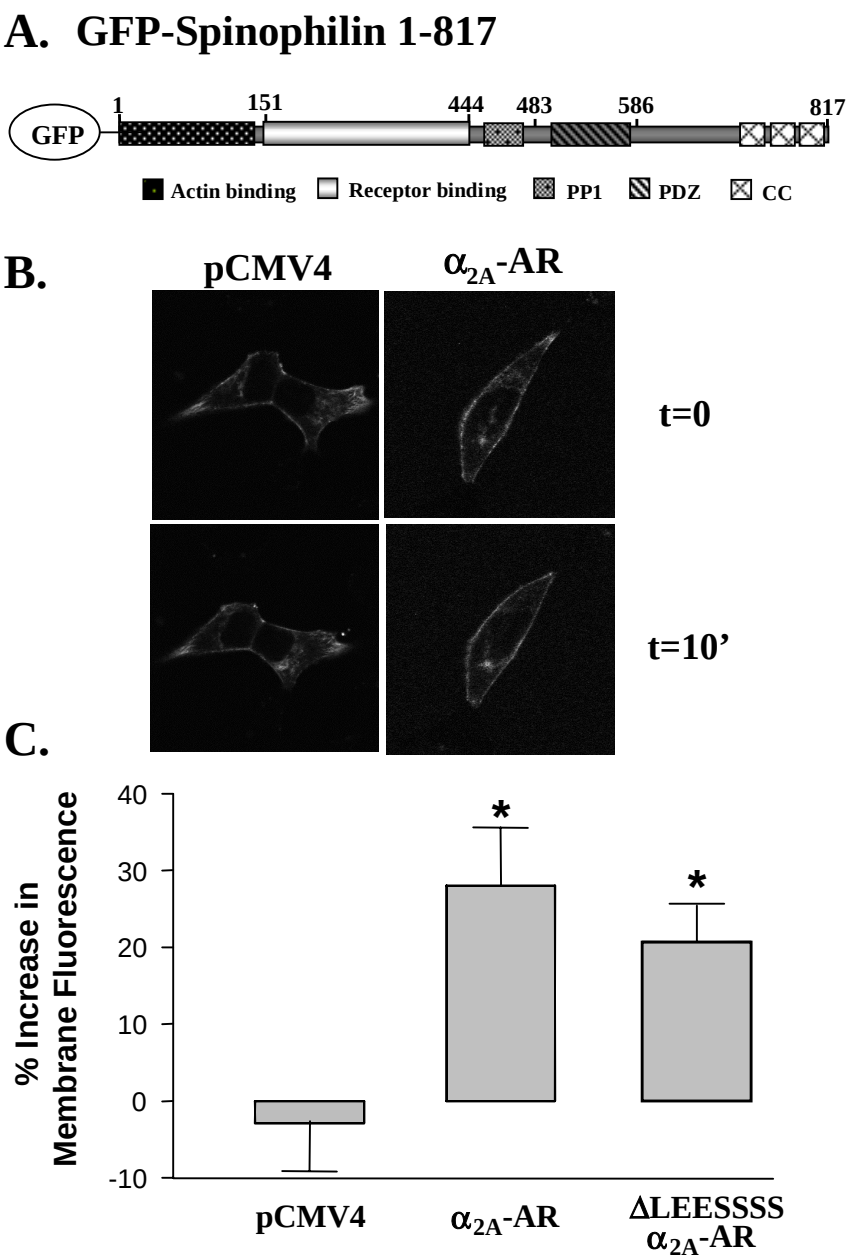

Fig 2.

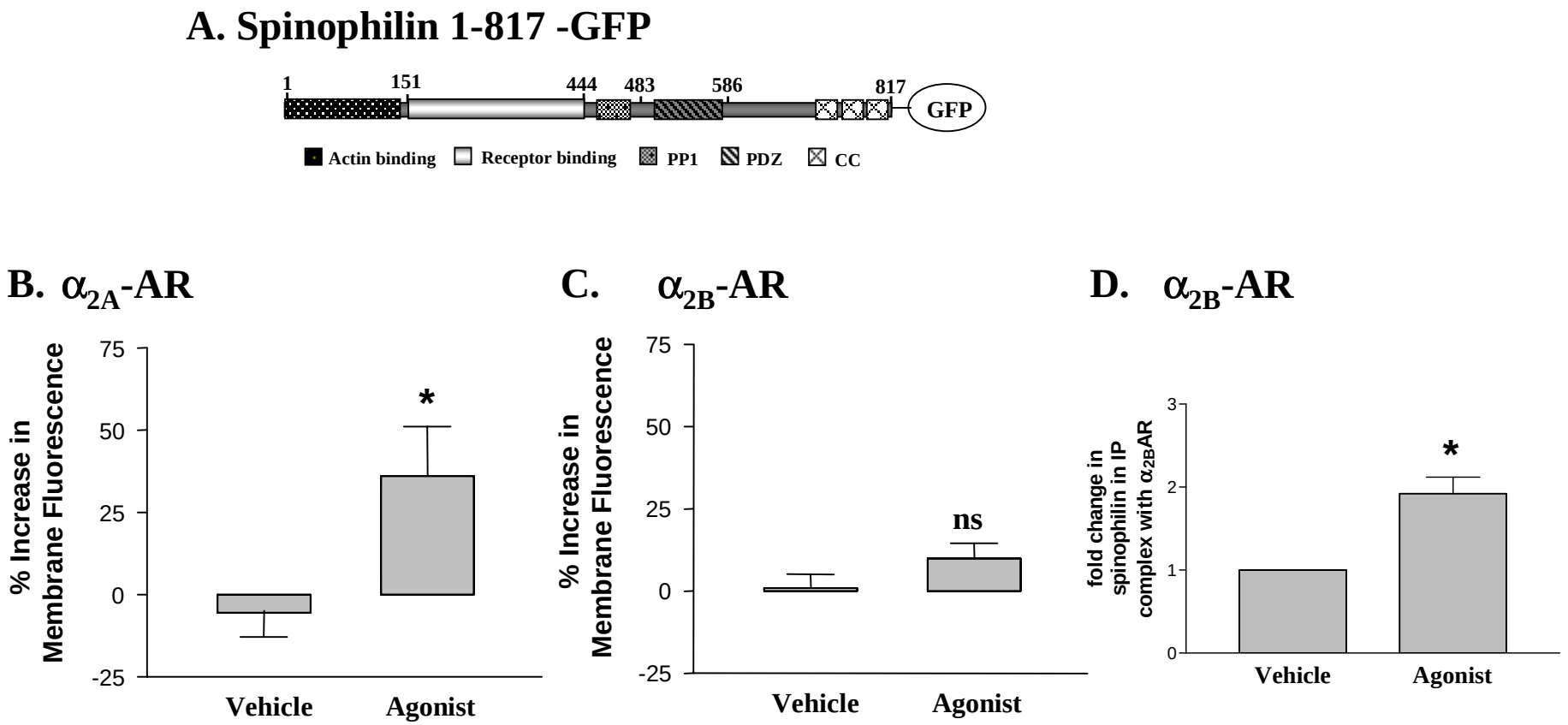

Fig 3.

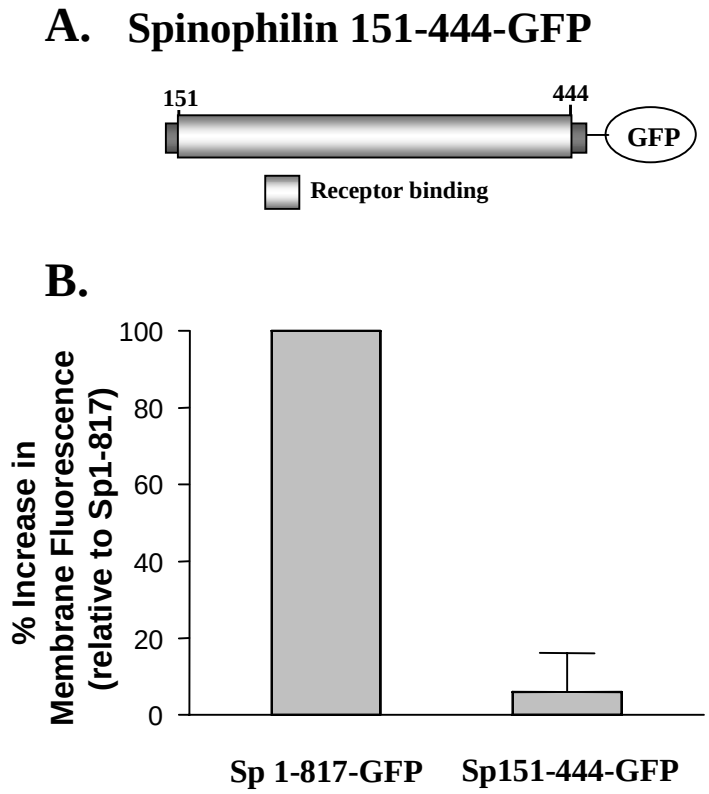

Fig 4.