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Microtubule-Dependent Regulation of α_{2B} Adrenergic Receptors in Polarized MDCKII Cells Requires the Third Intracellular Loop but Not G Protein Coupling

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ABSTRACT

Previous studies in cultured, polarized Madin-Darby canine kidney II (MDCKII) renal epithelial cells have demonstrated that the apical steady-state localization and delivery of the A_1 adenosine receptor is modified by disruption of the microtubule network with colchicine, whereas the basolateral localization and trafficking of the α_2-adrenergic receptors (α_2AR) are not; instead, the binding capacity of the α_{2B}AR, but not α_{2A}AR or α_{2C}AR subtypes, is increased in a time-dependent fashion. The present studies explore the molecular basis for this α_{2B}AR subtype-selective phenomenon. Colchicine selectively increased α_{2B}AR density at the cell surface, as determined by confocal microscopy, receptor binding, and surface biotinylation studies. The colchicine-induced increase in the functional density of the α_{2B}AR requires the third intracellular loop because the α_{2B}AR loop deletion (α_{2B}AR△i3) mutant did not show an increased receptor density after colchicine treatment. Furthermore, the colchicine-mediated increase in α_{2B}AR density is manifest only in polarized cells because colchicine treatment of nonpolarized MDCKII renal epithelial cells as well as simian kidney COSM6 and human embryonic kidney HEK293 cells did not effect an increase in α_{2B}AR density. Colchicine-dependent increases in α_{2B}AR density did not depend on functional coupling to G proteins, however, because pretreatment with pertussis toxin did not eliminate the effect of colchicine. These data indicate that microtubule-dependent regulation of α_{2B}AR density at the basolateral surface of polarized MDCKII cells requires the third intracellular loop of α_{2B}AR but not functional α_{2B}AR-G protein coupling.

The mechanisms by which G protein-coupled receptors (GPCRs) attain their localization in polarized epithelial cells are an important determinant of *trans*-epithelial function. Using polarized Madin-Darby canine kidney II (MDCKII) cells as a model system, we previously described the different trafficking itineraries for the three α_2-adrenergic receptor (AR) subtypes (Wozniak and Limbird, 1996). The α_{2A}AR and α_{2C}AR subtypes are directly delivered to the basolateral surface, whereas the α_{2B}AR is randomly delivered to both apical and basolateral surfaces. The α_{2B}AR achieves its steady-state basolateral enrichment due to its retention on that surface ($t_{1/2}$ = 10–12 h) compared with its rapid turnover on the apical surface ($t_{1/2}$ = 5–15 min). At steady state, the α_{2A}AR and α_{2B}AR subtypes are nearly exclusively on the basolateral surface, whereas a substantial fraction of the α_{2C}AR population remains in a cytoplasmic compartment (Wozniak and Limbird, 1996), corroborating earlier findings in nonpolarized cells (von Zastrow et al., 1993). Mutagenesis strategies, undertaken in detail for the α_{2A}AR subtype, suggest that the third intracellular loop of the α_{2A}AR is critical for retention of this subtype on the basolateral surface, but that targeting to the bilayer involves sequences or structures embedded in or near the bilayer (Keefer et al., 1994).

We previously had shown that the A_1 adenosine receptor (A_1AdoR), also coupled to G_i/G_o G proteins, is directly delivered to the apical surface of MDCKII cells and enriched there at steady state (Saunders et al., 1996; Saunders and Limbird, 1997). Studies of surface delivery of truncations of the α_{2A}AR or chimeras with the A_1AdoR suggest that multiple independent sequences exist in the bilayer of GPCRs to determine targeting of these seven transmembrane-spanning molecules in a hierarchical fashion to one versus another surface in polarized cells (Saunders et al., 1998).

Receptors and other cell surface proteins are not the only nonrandomly distributed molecules in polarized cells. The apical surface is undergirded by an actin-rich cytoskeletal network, whereas an ankryn-fodrin-rich cytoskeleton underlies the basolateral surface (Nelson and Hammerton, 1989). Furthermore, actin microfilaments, microtubules, and inter-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; MDCK, Madin-Darby canine kidney; AR, adrenergic receptor; A_1AdoR; A_1 adenosine receptor; HA, hemagglutinin; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; [^{125}I]PIC, *p*-[^{125}I]iodoclonidine; MAP, mitogen-activated protein.

mediate filaments, involved in vesicle and protein movement within all cells, have been particularly implicated in polarized trafficking and subsequent function of membrane-targeted molecules (Matter et al., 1990; Lafont et al., 1994; Arreaza and Brown, 1995). We previously have observed that disruption of microtubules with colchicine or nocodazole in polarized MDCKII cells paralleled a reduced apical delivery of A_1AdoR and an enrichment, or rerouting, of the A_1AdoR to the basolateral surface (Saunders and Limbird, 1997). In contrast, the same treatment had no impact on the random delivery of the α_{2B}AR subtype to both surfaces before selective retention on the basolateral surface. However, the amount of α_{2B}AR delivered to the surface was significantly enhanced in a time-dependent fashion when the microtubule network of MDCKII cells is depolymerized with colchicine (Saunders and Limbird, 1997). The present studies were undertaken to further explore the structural regions within α_{2B}AR that contribute to this unexpected subtype-selective increase in α_{2B}AR density and the mechanisms that may contribute to this phenomenon.

Experimental Procedures

Materials. [^{3}H]Methoxyinulin (125.6 mCi/g) was purchased from DuPont/NEN (Boston, MA). Protein A-purified 12CA5 monoclonal antibody was from the Berkley Antibody (Richmond, CA); Cy-3-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA); monoclonal anti-β-tubulin was from Amersham (Arlington Heights, IL); and rhodamine-conjugated phalloidin was from Molecular Probes (Eugene, OR). Colchicine and lumicolchicine were from Sigma Chemical Co. (St. Louis, MO).

Construction of α_{2B}AR△i3-TAG. The first nine amino acids after the initiating methionine of the α_{2B}AR evaluated in these studies encode a hemagglutinin (HA) epitope recognized by the commercially available monoclonal antibody 12CA5 (Berkley Antibody). The HA-epitope tagged, mutant α_{2B}AR with the third intracellular loop deletion was constructed with the Stratagene mutagenesis kit (La Jolla, CA). The construction of the HA-epitope-tagged α_{2B}AR has been described previously (Wozniak and Limbird, 1996). Purified oligonucleotides were generated to engineer two *Not*I sites in the third intracellular loop of the α_{2B}AR, one at nucleotide 639 and one at nucleotide 1062. Subsequently, nucleotide sequences encoding the majority of the third loop were excised via *Not*I restriction digest, and the remaining receptor sequence was ligated back together with the T4 DNA ligase. This resulted in a construct that encoded an α_{2B}AR mutant protein in which only the membrane proximal sequences of the third loop remained intact (total third loop length for the α_{2B}AR is 178 amino acids, whereas the third loop of the α_{2B}AR△i3 is 38 amino acids). In the α_{2B}AR△i3 structure, 14 amino acids in the proximal (amino terminal) and 14 amino acids into the distal (carboxy terminal) portion of the third cytoplasmic loop are retained because these regions have been demonstrated to be critical for G protein coupling (Wade et al., 1994; Eason and Liggett, 1996). The α_{2B}AR△i3 mutant was verified by dideoxy-DNA sequencing.

COSM6 Transfection and Cell Culture. This was done as previously described in Guyer et al. (1990). Briefly, transient transfection of simian kidney COSM6 cells (100-mm dishes plated at 1×10^6 cells/dish) with 10 μg of DNA by the DEAE-dextran method of transfection was used to assess the level of receptor expression with both immunocytochemistry (as described in "Steady-State Localization of GPCRs by Immunolocalization") and [^{3}H]yohimbine binding (as described in "Receptor-Binding Assays").

Development of Permanent Transformants of Human Embryonic Kidney (HEK)293 Cells. This was done as described previously in Schramm and Limbird (1999). HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in a 5% CO_2 incubator. Permanent transfectants were generated by lipofectamine-mediated cotransfection of the cells with plasmids containing the indicated receptors and a neomycin resistance gene. Cells that survived selection in medium containing 500 μg/ml G-418 were screened for expression of the expected receptor by binding of the radiolabeled α_2AR antagonist [^{3}H]rauwolscine. Clonal cell lines with varying levels of α_2AR expression were kept for further study. The experiments reported herein were performed on an α_{2B}AR expressing cell line that contains 2 to 4 pmol/mg of receptor binding.

Development of Permanent Transformants of MDCKII Cells. Permanent clonal cell lines of MDCKII cells were developed as described previously (Keefer and Limbird, 1993; Wozniak and Limbird, 1996). The clonal cell lines evaluated in the present study include TAGα_{2A}AR (25 pmol/mg and 7 pmol/mg protein), TAGα_{2B}AR (10 pmol/mg and 3 pmol/mg protein), and TAGα_{2C}AR(5 pmol/mg and 3 pmol/mg protein). The α_{2A}AR subtype was encoded by a porcine cDNA; the α_{2B}AR and α_{2C}AR subtypes by a rat cDNA.

Polarized Culture of MDCKII Cells and Functional Confirmation of Intact Monolayers. MDCKII cells were maintained as described previously (Keefer and Limbird, 1993). For polarity experiments, MDCKII cells were seeded at a density of 1×10^6 cells/24.5-mm polycarbonate membrane filter (Transwell chambers, 0.4-μm pore size; Costar, Cambridge, MA), and cultured for 5 to 8 days with medium changes every day. Before each experiment, the integrity of the monolayer was assessed by monitoring [^{3}H]methoxyinulin leak (Keefer et al., 1994).

Receptor-Binding Assays. MDCKII particulate preparations were prepared essentially as described in Keefer and Limbird (1993). Briefly, all MDCKII cell lines were grown in Transwell culture (except when stated otherwise) and allowed to polarize for 7 to 8 days. Binding assays were performed on membranes harvested from these cells treated (or not) with varying agents examined in this study. Cells were harvested into a lysis buffer: 15 mM Tris-HCl, 5 mM EGTA, 5 mM EDTA, pH 8.0, containing 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 100 μg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride.

Assessment of Antagonist Binding. The pellet resulting from two washes in lysis buffer followed by centrifugation at 30,000*g* was resuspended in 900 μl of antagonist binding buffer (20 HEPES, 25 glycine/glycine, 100 NaCl, 5 EGTA, pH 7.4). COSM6 cells and HEK293 cell membranes were prepared similarly. For all the cell types, [^{3}H]rauwolscine binding was performed in 12 × 75-mm polypropylene tubes containing 3 nM [^{3}H]rauwolscine (diluted in water) in the absence (total binding) or presence (nonspecific binding) of 10 μM phentolamine, an α_2AR antagonist. Incubations were for 30 min at 25°C, and were terminated by the addition of 3.5 ml of ice-cold 25 mM glycyl glycine buffer, pH 8.0, and filtration through Whatman GF/B glass microfiber filters.

Assessment of Agonist Binding. Membranes from clonal cell lines expressing wild-type or mutant receptors were harvested and washed in lysing buffer but resuspended and incubated in an agonist buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, and 5 mM EGTA, pH 8.0. Incubations were for 30 min at 25°C and contained 100 μg of membrane protein, 0.9 nM *p*-[^{125}I]iodoclonidine ([^{125}I]PIC) agonist radioligand (~160,000 cpm/100-μl incubation). The incubation was terminated by vacuum filtration and the GF/B filters were counted in a Beckman 4000 gamma counter.

Guanine Nucleotide Sensitivity of Radioligand Agonist Binding as a Measure of α_{2B}AR-G Protein Coupling. The addition of Gpp(NH)p, a hydrolysis-resistant GTP analog, to membrane preparations containing G protein-coupled receptors typically disrupts receptor/G protein coupling, shifting the receptor from a higher-affinity state for agonists (functional receptor-G protein coupling) to a lower-affinity state for agonists (receptor functionally dissociated from G protein) (Stadel et al., 1980). Consequently, the ability of guanine nucleotides to decrease the detectability of radiolabeled agonist binding is an indirect measure of the existence of guanine

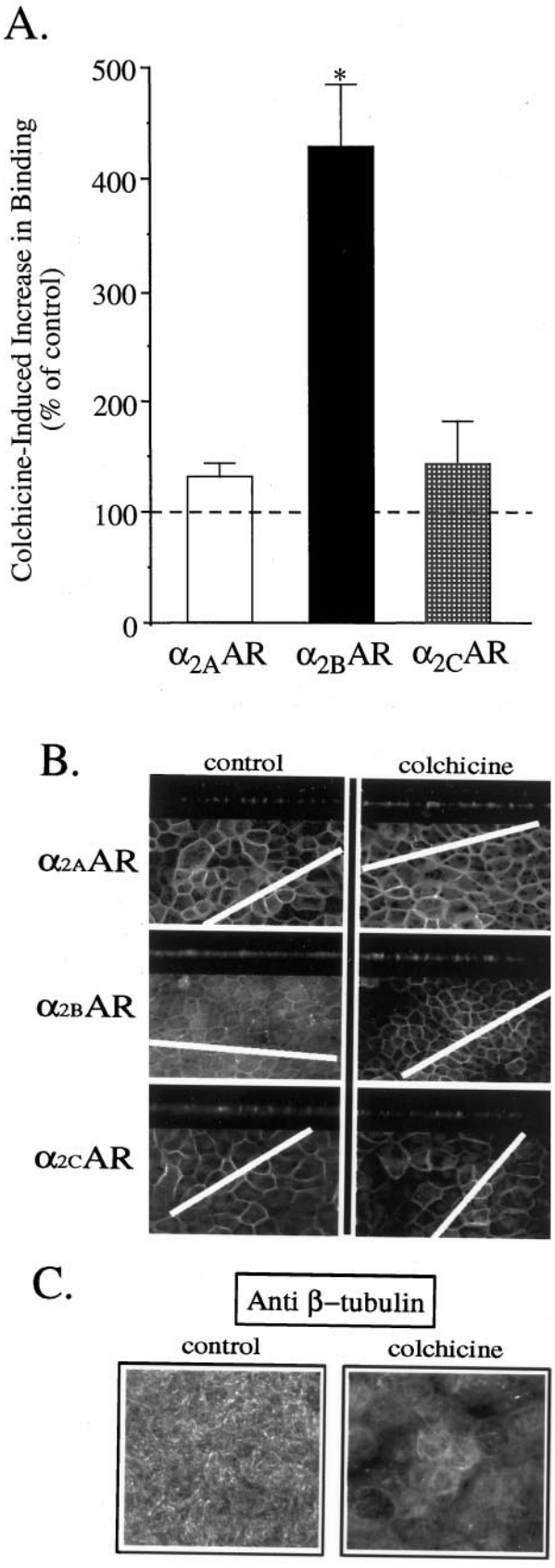

Fig. 1. Colchicine increases density of the α_{2B}AR in a subtype selective manner. A, functional density of the α_{2B}AR subtype is increased by colchicine in polarized MDCKII cells as assessed by [^{3}H]rauwolscine saturation binding to membrane preparations derived from these cells expressing heterologous α_2AR subtypes and grown in 75-mm Transwell culture. Binding density was assessed after culture in the presence or absence of 10 μM colchicine (15 h). *$P < .05$, as assessed by one-way ANOVA. The increase in α_{2B}AR binding resulted from an increase in receptor density (B_{max} = 1 ± 0.06 pmol receptor binding/mg protein and K_D = 1.86 ± 0.18 nM (control) versus 2.96 ± 0.67 pmol receptor binding/mg protein and K_D = 2.23 ± 0.16 nM following colchicine treatment. B, confocal immunofluorescence images of α_2AR subtypes in polarized MDCKII cells incubated in the presence or absence of 10 μM colchicine treated as in (A). The cells were detected on their Transwell filter supports, as described in "Methods." C, untransfected (parental) polarized MDCKII cells were treated or not with colchicine as in (A). and then stained with an anti-β-tubulin monoclonal antibody to confirm that the colchicine treatment used was effective in disrupting microtubule structure in these cells.

nucleotide-sensitive high-affinity receptor-agonist interactions (Williams and Lefkowitz, 1977; Gerhardt et al., 1990). To evaluate the ability of Gpp(NH)p to modulate radiolabeled agonist binding, [^{125}I]PIC incubations were performed in the absence (control) or presence of increasing concentrations of Gpp(NH)p. When evaluated, pertussis toxin sensitivity of receptor-GTP interactions was determined by incubation of MDCKII cells overnight with 200 ng of pertussis toxin/ml of culture medium (10 ml of medium/100-mm dish) (Keefer and Limbird, 1993; Ceresa and Limbird, 1994). To assess the relative fraction of receptors achieving high-affinity agonist binding due to receptor-G protein interactions by different receptor structures or under different incubation conditions, the quantity of guanine nucleotide-sensitive [^{125}I]PIC binding (picomoles per milligram) to total receptor density of [^{3}H]rauwolscine binding (picomoles per milligram) can be compared.

Treatment of Cells with Colchicine. Colchicine is an irreversible microtubule-disrupting drug that binds slowly to soluble tubulin heterodimers, reducing them to large aggregates and rendering them incapable of polymerizing for microtubule growth. Incubations with 10 μM colchicine were performed for 15 h as previously described (Saunders and Limbird, 1997). Immunocytochemical analysis of treated cells with an anti-β-tubulin antibody confirmed that colchicine treatment of cells had indeed disrupted the microtubule network, as seen in Fig. 1C.

Steady-State Localization of GPCRs by Immunolocalization. Immunostaining of cells grown in Transwell culture was performed as described previously (Saunders et al., 1996; Saunders and Limbird, 1997) with the following concentrations of primary antibody: a 1:50 dilution of 12CA5 primary antibody, purified as described previously (Wozniak and Limbird, 1996), for the localization of hemagglutinin epitope-tagged GPCRs (Keefer and Limbird, 1993). The antibody-containing buffers and wash buffers contained 0.1% Triton X-100 to permit detection of epitope on the cell surface and in the cell interior. Treatment with the secondary Cy3-conjugated donkey anti-mouse IgG (1:200) was performed as described in Saunders and Limbird (1997). Samples were visualized by confocal microscopy on a Zeiss Axiovert 135 Micro System LSM (Oberkochen, Germany). The samples were first visualized in the *xy* plane, and then in the *xz* plane. In the images shown, the bottom three-fourths represent the *xy* plane, the conventional view of the cells as one looks down on them. The white line that is shown in the *xy* plane confocal images indicates where the laser took a cross-section of the cells to generate the z scan. The top one-fourth of the image represents the *xz* plane (or z scan), the cortical section perpendicular to the plane of the cell layer. Images were analyzed with Showcase software on a Silicon Graphics (Mountain View, CA) iris indigo workstation.

Steady-State Localization of α_{2B}AR and α_{2B}AR△i3 in MDCKII Cells: Biotinylation and Photoaffinity Labeling. The previously described method (Wozniak and Limbird, 1996) for quantitating the apical versus basolateral (versus intracellular) distribution of the wild-type and mutant α_{2B}AR in polarized MDCKII cells was biotinylation of the apical versus the basolateral surface of cells grown in Transwell culture, photoaffinity labeling of the functional α_{2B}AR in harvested

membranes, detergent extraction, and isolation of biotinylated receptors via streptavidin-agarose chromatography. The cell lines grown in Transwell culture were biotinylated on ice for 30 min with sulfo-NHS biotin to covalently label the primary amines of the α_{2B}AR. Following membrane preparation, the α_{2B}AR-expressing cell lines (both wild type and α_{2B}AR△i3) were covalently modified with the photoactivatable α_{2B}AR-selective ligand [^{125}I]Rau-AzPEC for 1 h at 15°C in the dark. Photolabeling not attributable to receptor binding was determined in parallel incubations carried out in the presence of 10 μM phentolamine, an α-adrenergic receptor antagonist. The photoaffinity-labeled receptors were then extracted in RIPA buffer. Streptavidin-agarose chromatography was used to isolated the biotinylated (and now photoaffinity-labeled) molecules. The fraction of biotinylated, photoaffinity-labeled α_2AR present on the apical versus basolateral surface was determined following SDS-polyacrylamide gel electrophoresis followed by autoradiography. The films were then scanned and imported into Adobe Photoshop.

Mitogen-Activated Protein (MAP) Kinase Stimulation. Activation of MAP kinase was assessed as previously described for HEK293 cells (Schramm and Limbird, 1999) with a few alterations. Clonal MDCKII cell lines were plated on 24-mm Transwell filters (Transwell chambers, 0.4-μm pore size; Costar, Cambridge, MA) at confluency. The cells were then serum-deprived overnight. On the day of the experiment, the cells in Transwell culture were moved to a plate warmer kept at 37°C, and the medium was replaced with fresh serum-free DMEM. The α_2AR agonist UK-14304 was added for 2 or 10 min at all final concentration of 1 μM directly to the medium on the cells and very gently swirled to mix. An equal volume of medium was added to the control well. After the indicated times, the cells were washed once with Dulbecco's PBS containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$, then lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% w/v SDS; 10% glycerol; 50 mM dithiothreitol) supplemented with 1 mM sodium orthovanadate (Sigma Chemical Co.), 10 U/ml leupeptin (Sigma Chemical Co.), and 10 U/ml aprotinin (Bayer, Kankakee, IL). The lysates were transferred to an Eppendorf tube on ice. When all samples were collected, they were sonicated for 20 s, then placed in a heating block at 95°C for 5 min. The lysates were then centrifuged in a microcentrifuge at room temperature for 5 min to remove debris. The supernatants were assayed in Bio-Rad's protein assay for relative protein concentration, and equivalent amounts of protein were loaded on a 10% SDS-polyacrylamide gel for electrophoresis. The gel was run for 160 mAmp-hours, then transferred overnight onto nitrocellulose in transfer buffer (20% methanol, 0.19 M glycine, 25 mM Tris base) at 33 mV. MAP kinase activation was evaluated with an antibody that recognizes dually phosphorylated (Thr/Tyr) MAP kinase (catalog no. V6671; Promega, Madison, WI) and normalized to total MAP kinase with an antibody that recognizes MAP kinase regardless of its phosphorylation state (catalog no. 9102; NEB, Beverly, MA). To assess activated MAP kinase content, the nitrocellulose blot was incubated in blocking buffer (1× Tris-buffered saline; 0.1% Tween 20; 5% w/v nonfat dry milk) for 1 h at room temperature, then probed with Promega's rabbit polyclonal antibody to dually phosphorylated MAP kinase, diluted 1/500 in blocking buffer, for 1 h at room temperature. The blot was washed three times for 5 min each with Tris-buffered saline/Tween 20 (2.42 g/l Tris base, 8.0 g/l NaCl, 0.1% Tween 20, pH 7.6) then probed with donkey anti-rabbit horseradish peroxidase-linked secondary antibody (1/2000 dilution in blocking buffer) (Amersham) for 1 h at room temperature. The wash protocol was repeated, and the immunoreactive bands were detected by enhanced chemiluminescence (Amersham) The blots were then stripped with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) for 30 min at 65°C, and reprobed with antibody to total MAP kinase (NEB) at a 1/500 dilution in blocking buffer overnight at 4°C, followed by donkey anti-rabbit secondary antibody as described above. The enhanced chemiluminescence images were scanned into Adobe Photoshop with a UMAX Astra 600 scanner.

Results

Colchicine Selectively Increases Receptor Density of α_{2B}AR Subtype at Cell Surface. As seen in Fig. 1A, overnight treatment with 10 μM colchicine dramatically increased the density of α_{2B}AR capable of binding ligand, but did not change functional receptor density significantly for the α_{2A}AR or α_{2C}AR subtypes. As shown previously, increased α_{2B}AR binding was observed for colchicine and, to a lesser extent, for nocodazole, another microtubule-disrupting agent (Saunders and Limbird, 1997). The increase in receptor density was time dependent; it was not noted before 2 h and displayed a maximal effect at 15 h. Changes in α_{2B}AR binding were not observed with γ-lumicolchicine, a chemical analog of colchicine that does not disrupt microtubules (data not shown).

As shown in Fig. 1B, the increase in functional α_{2B}AR-binding capacity was paralleled by a detectable increase in receptor fluorescence intensity on the lateral subdomain of polarized MDCKII cells when α_{2B}AR-expressing cells were pretreated with colchicine. This intense lateral staining was observed in conjunction with a decrease in the intracellular staining for the α_{2B}AR. In contrast, no quantitative or qualitative change in the staining profiles for the α_{2A}AR or α_{2C}AR was observed following colchicine treatment of polarized MDCKII cells (Fig. 1.B). The observation that the increase in the relative surface to intracellular distribution of the α_{2B}AR subtype was not shared by the α_{2C}AR subtype, which has a significant intracellular density, is consistent with previous findings that the intracellular compartments of the α_{2B}AR and α_{2C}AR subtypes are functionally distinct (von Zastrow et al., 1993).

Saturation-binding analyses revealed that the increase in α_{2B}AR binding detected at steady state was due to an increase in maximal receptor density with little change in α_{2B}AR receptor affinity for the radiolabeled antagonist [^{3}H]rauwolscine (see legend to Fig. 1A). Radioligand-binding assays with the hydrophobic antagonist [^{3}H]rauwolscine measure both surface accessible and intracellular receptors, whether performed in intact cell incubations or, as in these experiments, in broken cell assays (Fig. 1A). However, the disproportionate increase in receptor density we detect is an increase in surface receptor binding, as evidenced not only by the changes in relative fluorescence intensity of lateral α_{2B}AR (Fig. 1B) but also by the access of the α_{2B}AR to surface-accessible biotinylation reagents, before (Fig. 2C) or after colchicine treatment (data not shown). The increase in receptor density is paralleled by an increased synthesis and delivery of the α_{2B}AR to the basolateral surface after colchicine treatment (Saunders and Limbird, 1997), perhaps because the removal of an inhibitory microtubule network by colchicine allows more vesicles carrying receptor to reach the cell surface.

Colchicine-Induced Increase in Functional Density of α_{2B}AR Subtype Requires Third Intracellular Loop. Because the third intracellular loop of all three α_2AR subtypes interacts with other proteins, including 14–3-3 ζ (Prezeau et al., 1999), we wanted to see if the third intracellular loop of the α_{2B}AR was required to detect the colchicine-induced increase in receptor density. We created a mutant α_{2B}AR, α_{2B}AR△i3, in which the third intracellular loop was deleted, except for the membrane proximal portions responsible for coupling to G pro-

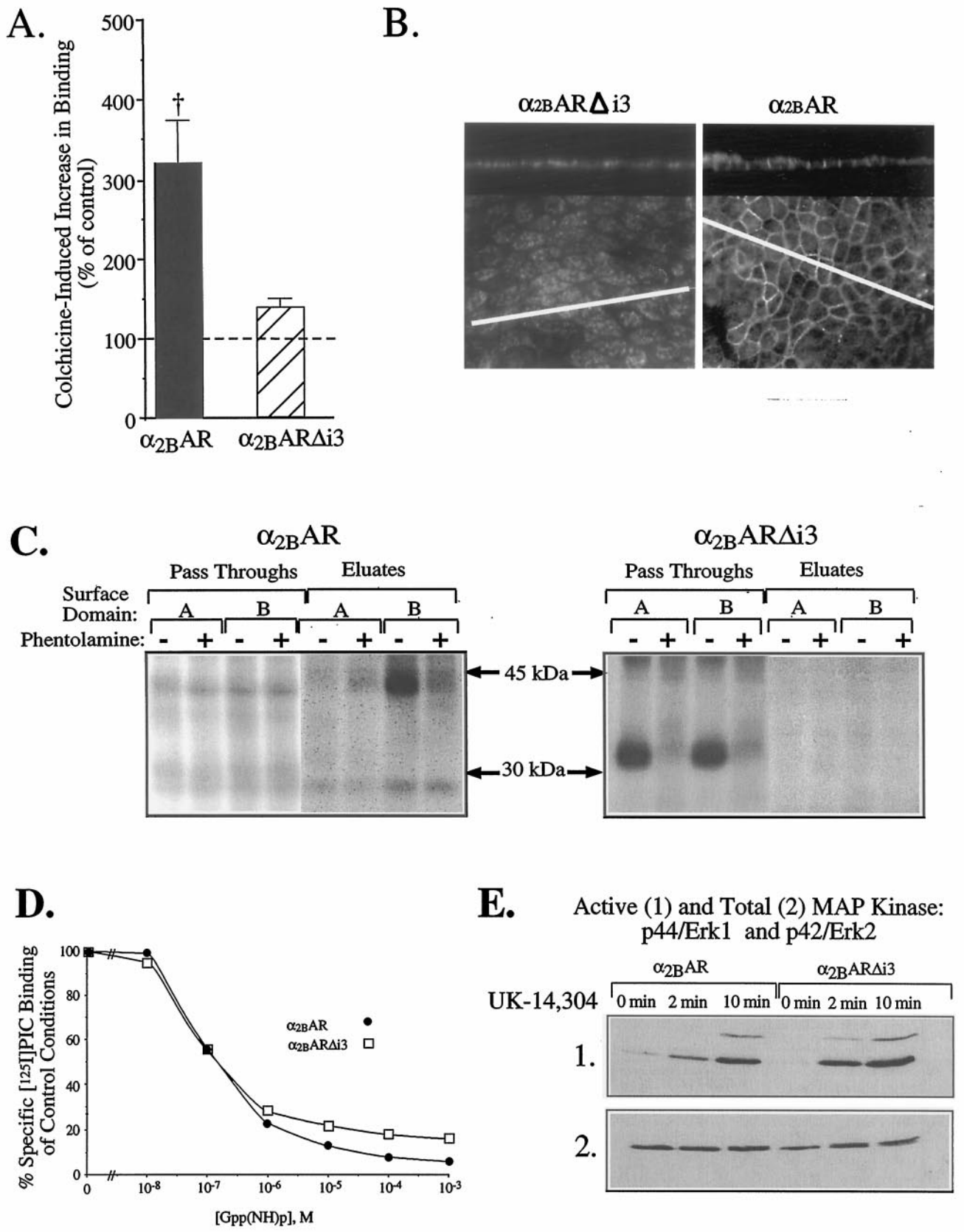

Fig. 2. The colchicine-induced increase in the functional density of the α_{2B}AR subtype requires the third intracellular loop. A, radioligand binding of [^{3}H]rauwolscine to α_{2B}AR and α_{2B}AR△i3 was assessed following treatment (or not) with 10 μM colchicine for 15 h as described in Fig. 1. The data shown are means ± S.E. (n = 3). *$P < .05$, as assessed by Student's t test. B, confocal images for the α_{2B}AR and α_{2B}AR△i3 as described in "Methods." The staining pattern of the α_{2B}AR△i3 expressed in MDCKII cells was compared with that of the wild-type α_{2B}AR. MDCKII cells expressing α_{2B}AR△i3 were grown in Transwell culture for 4 to 7 days to attain a polarized phenotype. Once the monolayer integrity was confirmed by the [^{3}H]methoxyinulin

teins (Saunders and Limbird, 1997). As seen in Fig. 2A, the colchicine-mediated increase in α_{2B}AR density was not observed in the absence of the third intracellular loop. Confocal microscopy images of immunofluorescence staining revealed that this mutant α_{2B}AR△i3 is localized predominantly intracellularly (Fig. 2B), as is confirmed with surface biotinylation and photoaffinity-labeling strategies (Fig. 2C). Surface biotinylation followed by photoaffinity labeling of the α_{2B}AR with the α_2AR antagonist [^{125}I]Rau-AzPEC demonstrates that, for the wild-type α_{2B}AR, the receptor is predominantly in the streptavidin eluates, meaning that the binding detected is virtually exclusively occurring on the biotinylated cell surface. In contrast, the photoaffinity-labeled α_{2B}AR△i3 is in the pass throughs of streptavidin-agarose chromatography, indicative of little or no α_{2B}AR△i3 on the cell surface and therefore little or no biotinylation of the α_{2B}AR△i3 by membrane impermeant reagents (Fig. 2C).

The ability of the intracellular α_{2B}AR△i3 to bind ligand, as demonstrated by the lack of deposition of detergent-extracted α_{2B}AR△i3 to streptavidin agarose, is an important finding because it is the first direct documentation that intracellular α_2AR can bind ligands. Interestingly, despite its almost exclusive intracellular localization, the α_{2B}AR△i3 still bound agonist (Fig. 2D) and antagonist (Fig. 2A), coupled to G proteins (Fig. 2D), and activated MAP kinase activity in a manner comparable to the wild-type α_{2B}AR (Fig. 2E). Guanine nucleotide sensitivity of receptor affinity for agonist, one measure of functional receptor-G protein coupling, is most sensitively assessed by examining the ability of quanine nucleotides, such as the hydrolysis-resistant Gpp(NH)p, to decrease detectable radiolabeled agonist binding, because Gpp(NH)p induced lower-affinity agonist-receptor interactions cannot be trapped by filtration assays (Williams and Lefkowitz, 1977; Gerhardt et al., 1990), as shown in Fig. 2D. This guanine nucleotide sensitivity characteristic of the wild-type α_{2B}AR also is observed for the α_{2B}AR△i3. Because heterotrimeric G proteins have been detected in intracellular compartments (Muntz et al., 1992), perhaps this receptor-G protein coupling of an intracellular receptor is not surprising. Previous studies (Wade et al., 1994; Eason and Liggett, 1996) have mapped the G protein-coupling interface to amphipathic helices at the base of transmembrane domains 5 and 7, and these are still present in this mutant structure. Perhaps somewhat surprising was the ability of α_{2B}AR△i3 to activate MAP kinase in polarized MDCKII cells because our own studies (Schramm and Limbird, 1999) have shown that activation of MAP kinase terminates on receptor internalization and is sustained under conditions (elevated K^+) that block agonist-elicited redistribution of the wild-type α_{2B}AR. Nonetheless, these findings do affirm that the structure of the α_{2B}AR△i3 resembles that of the wild-type α_{2B}AR sufficiently to be able to bind ligand, activate G proteins, and elicit signaling.

Colchicine-Induced Increase in α_{2B}AR Density Was Observed Only in Polarized Cells. The cytoskeletal ultrastructure of polarized cells is very different from that of nonpolarized cells. Two main groups of microtubule networks exist in polarized epithelial cells: a randomly organized group, arranged around the apical area of the cell, and a polarized network that runs along the lateral sides of the cells, with the minus ends of the microtubules facing the apical apex, and the plus ends facing the basal side. Because this disparate arrangement is not present in nonpolarized cells, we evaluated whether the colchicine-induced increase in α_{2B}AR density also could manifest itself in nonpolarized cells, both those which had the potential to polarize under the appropriate culture conditions (MDCKII cells), and those that do not possess the potential to do so, such as COSM6 and HEK293 cells. As seen in Fig. 3, the ability of colchicine to increase α_{2B}AR density is most readily detected in MDCKII cells grown in Transwell culture to a functionally and morphologically polarized state (Nelson and Veshnock, 1987). In Transwell culture, both the basal surface, via the nitrocellulous filter, and the apical surface have direct access to nutrients. When the cells are plated densely on plastic, close apposition of the cells forces the cells to form a tight monolayer at the lateral sides of the cells in a pseudopolarized phenotype, but the basal surface does not readily get access to medium. As seen in Fig. 3, the colchicine-mediated increase in receptor density is diminished in MDCKII cells grown on plastic compared with cells grown in Transwell culture, i.e., when greater polarization is achieved. Alternatively, when MDCKII cells are plated sparsely on plastic (i.e., 20–40% confluent), there is no detectable effect of colchicine on α_{2B}AR density, in parallel with the absence of MDCKII cell polarization under these conditions (Nelson and Veshnock, 1987). The necessity of a polarized cell environment to detect colchicine-evoked increases in α_{2B}AR density is further evidenced by the lack of a colchicine-induced effect on steady state α_{2B}AR density in either COSM6 cells transiently expressing the α_{2B}AR, or in HEK293 cells permanently expressing the α_{2B}AR (Fig. 3).

Colchicine-Induced Increase in α_{2B}AR Density Is Not Dependent on Functional Coupling to G Proteins. We explored the possibility that the colchicine-induced in-

were grown in Transwell culture for 4 to 7 days to attain a polarized phenotype. Once the monolayer integrity was confirmed by the [^{3}H]methoxyinulin leak assay, the cells were fixed, permeabilized, and stained with the Babco 12CA5 monoclonal antibody directed against the 9 amino acid HA tag at the N terminus of the receptor, followed by staining with donkey anti-mouse Cy-3 secondary antibody. The Transwell filter with the adherent cells was then mounted onto a glass slide and visualized on a Zeiss Axiovert confocal microscope. The fluorescence pattern was compared with that of the wild-type α_{2B}AR. C, streptavidin-agarose resolution of detergent-solubilized α_{2B}AR and α_{2B}AR△i3 extracted from polarized MDCKII cells after surface biotinylation. The α_{2B}AR is enriched on the cell surface, and thus retained on and eluted from streptavidin-agarose resin after surface biotinylation. In contrast, the mutant α_{2B}AR△i3 is mostly intracellular, and therefore is not retained on streptavidin-agarose because intracellular receptor does not have access to biotin, such that the bands representing photoaffinity-labeled receptor are present in the pass throughs only and not in the streptavidin eluates. The authenticity of the band migrating at ~45 kDa (the wild-type α_{2B}AR) and ~30 kDa (the α_{2B}AR△i3) is verified by the lack of a band at these molecular masses when the photoaffinity-labeling reaction with membranes occurs in the presence of the antagonist phentolamine, which competes for receptor access of [^{125}I]Rau-AzPEC and thus protects against photoaffinity labeling. D, guanine nucleotide regulation of radiolabeled agonist, [^{125}I]*para*-iodo-clonidine, binding to membranes derived from MDCKII cells. The data shown are means ± S.E. from three independent experiments. E, activation of MAP kinase by the α_2AR agonist UK-14304 in polarized MDCKII cells expressing the wild-type α_{2B}AR or α_{2B}AR△i3. Activated MAP kinase (gel 1) was detected with the Promega antibody (as described in "Methods") directed against the dually phosphorylated Erk1 and Erk2. Total MAP kinase was shown to confirm that we have equal loading of protein on the gel. When stimulated with the α_2AR agonist, UK-14,304, as a function of time, untransfected MDCKII cells did not reveal any MAP kinase activation as revealed by a blank film (data not shown). The data are from one experiment representative of four independent experiments.

crease in α_{2B}AR density might require active coupling of the receptor to G proteins because multiple, independent studies have detected α- (Roychowdhury et al., 1999) and $\beta\gamma$- (Carlson et al., 1986; Roychowdhury and Rasenick, 1997) subunits of G proteins associated with the cytoskeleton. Overnight treatment of the MDCKII cells expressing α_{2B}AR with pertussis toxin (200 ng/ml), under conditions previously established to maximally ADP-ribosylate G_i (Keefer and Limbird, 1993), did not eliminate the effect of colchicine to increase α_{2B}AR density, as seen in Fig. 4A. The incubation with pertussis toxin was sufficient to uncouple the α_{2B}AR from G proteins, as reflected by the loss of guanine nucleotide modulation of receptor affinity for the radiolabeled agonist [^{125}I]PIC in pertussis-toxin treated preparations (Fig. 4B) and the decrease in the ratio of [^{125}I]PIC/[^{3}H]rauwolscine binding to that detected in preparations from control cells incubated in the presence of Gpp(NH)p. These findings demonstrate that functional coupling of the α_{2B}AR to pertussis toxin-sensitive G proteins is not required for colchicine-induced increases in α_{2B}AR density at the cell surface. Because the low molecular weight GTP-binding protein rho has been shown to be involved in cytoskeletal protein reorganization in response to extracellular signals (Takaishi et al., 1997), we examined whether pretreatment of cells with 10 μg/ml botulinum C3 exoenzyme, which ADP ribosylates and blocks its function, altered colchicine-induced increases in α_{2B}AR density. Botulinum toxin had no effect on the ability of colchicine to modulate α_{2B}AR density (data not shown).

Discussion

The study of the mechanisms that govern trafficking of receptors and signal transduction molecules is a rapidly emerging field, particularly in polarized cells where the correct localization of G protein-coupled receptors at polarized cell surfaces, for example, is crucial for the appropriate vectorial functioning of the cell. The polarized cytoskeleton plays an important role in the trafficking and eventual localization of the membrane proteins at their cell surface domain. The cytoskeleton has been described as viscoelastic: it provides a continuum of mechanical coupling throughout the cell that fluctuates as a function of the remodeling of the cytoskeleton (Janmey, 1998). These mechanical influences include changes in ion channel activity at the plasma membrane and propagation of mechanical stresses from the plasma membrane to the cytoplasm. The actin filaments and the microtubule network that comprise the major parts of the cytoskeleton have different but sometimes sequential functions in the trafficking of proteins in their target cells. Actin filaments are involved in cell polarity (Molitoris, 1997), endocytosis (Hirasawa et al., 1998), exocytosis, and translocation (Fincham et al., 1996). Microtubules are involved in two-way trafficking between the endoplasmic reticulum and the Golgi compartment (Rahkila et al., 1997), between the endoplasmic reticulum and plasma membrane (Robin et al., 1995), in signaling molecule processing and/or targeting (Thissen et al., 1997), and in some endocytic pathways involving late endosomes (Durrbach et al., 1996; Faigle et al.,

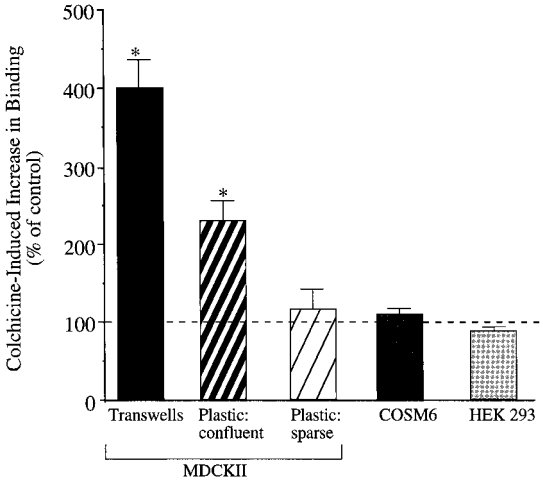

Fig. 3. Detectable colchicine-induced increases in α_{2B}AR density parallel the polarization of renal epithelial cells. Heterologously expressed α_{2B}AR was expressed in different renal cell types, including MDCKII, COSM6, and HEK293. The MDCKII and HEK293 cell lines were permanent expressing cell lines, whereas COSM6 cells transiently expressed α_{2B}AR. The number of repetitions of the experiments in various cell backgrounds was MDCKII Transwells (n = 6), MDCKII plastic confluent (n = 4), MDCKII plastic sparse (n = 4), COSM6 (n = 3), and HEK293 (n = 3). Each binding assay in each experiment was performed in triplicate. *$P <$ .05, as assessed by one-way ANOVA.

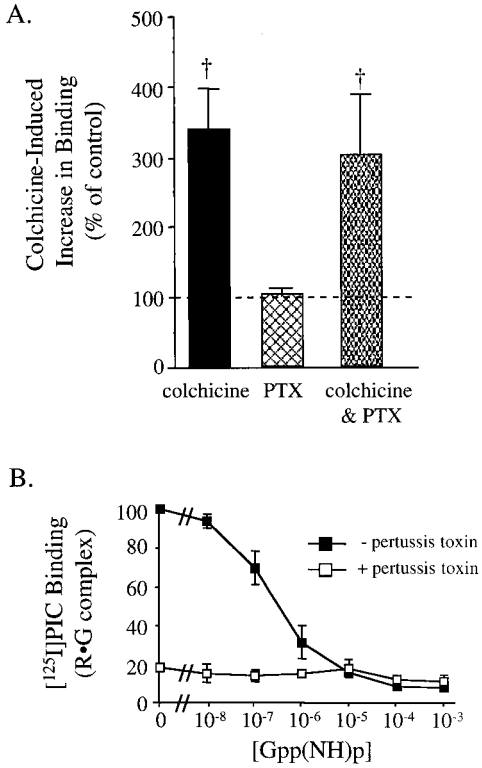

Fig. 4. Colchicine-induced increases in α_{2B}AR density are not dependent on receptor coupling to pertussis toxin-sensitive G proteins. A, radioligand binding of the heterologously expressed α_{2B}AR in polarized MDCKII cells grown on 75-mm Transwells was assessed in the presence of 10 μM colchicine (15 h) with the radiolabeled antagonist [^{3}H]rauwolscine, as described in "Methods." B, pertussis toxin treatment, used to evaluate the role of G_i on colchicine-mediated α_{2B}AR density increases, was effective in disrupting α_{2B}AR-G_i functional coupling, as performed in side-by-side experiments with MDCKII cells treated as in (A). [^{125}I]PIC agonist binding, an indirect measure of receptor-G protein coupling, as performed as described in "Methods." Disruption of α_{2B}AR-G protein coupling, either by incubation with Gpp(NH)p or prior treatment of cells with pertussis toxin, eliminates high-affinity agonist binding, such that the detectability of [^{125}I]PIC binding via vacuum filtration is eliminated. The data shown represent means ± S.E. from three independent experiments.

1998). Moreover, microtubules have been shown to be critical for the internalization of some GPCRs, such as the complement receptor (Allen and Aderem, 1996), whereas other GPCRs, such as the α_{1B}AR, instead require an intact actin filament network for internalization (Hirasawa et al., 1998). What has emerged over the past two decades is an understanding that the role of the cytoskeletal components can be different for the same protein in a variety of different cell backgrounds, and conversely, vary for similarly related proteins in the same cell type.

We are interested in elucidating the mechanisms conferring basolateral or apical localization of GPCRs in polarized cells. We previously have shown that the basolateral localization of the three α_2AR subtypes is not dependent on an intact microtubule network, i.e., depolymerization of microtubules with colchicine or nocodazole does not perturb their basolateral orientation in MDCKII cells (Saunders and Limbird, 1997). In contrast, the preferential apical targeting and localization of the A_1AdoR in renal epithelia (Saunders et al., 1996) is microtubule dependent because depolymerization of the microtubule network leads to preferentially more A_1AdoR delivered to and localized at the basolateral surface (Saunders and Limbird, 1997). In studying the trafficking of these GPCRs in the presence of cytoskeletal disrupting agents, we observed that 2- to 4-fold more α_{2B}AR was delivered to the cell surface in the presence of colchicine, based on metabolic labeling and surface biotinylation studies (Saunders and Limbird, 1997), whereas the surface delivery and steady-state density of the α_{2A}AR and α_{2C}AR subtypes was not affected by the same concentrations of colchicine. The aim of these studies was to further explore the mechanism for this α_{2B}AR subtype-specific colchicine-dependent increase in receptor density.

The observation of increased receptor delivery to the cell surface in the presence of colchicine from our earlier work (Saunders and Limbird, 1997) is consistent with the increased immunofluorescence intensity at the cell surface (Fig. 1B) observed in the presence of colchicine and the increase in functional binding capacity (Fig. 1A). The increased fluorsecence intensity at the cell surface for the α_{2B}AR subtype was paralleled by a decrease in the intracellular labeling characteristic of α_{2B}AR detected and quantified in confocal micrographs (Saunders and Limbird, 1997). In contrast, colchicine treatment did not lead to a decrease in intracellular fluorescence intensity for the α_{2C}AR subtype in the presence of colchicine (Fig. 1A), supporting previous interpretations that the intracellular pools of α_{2B}AR and the α_{2C}AR are morphologically and functionally distinct (von Zastrow et al., 1993; Wozniak and Limbird, 1996).

The selective effect of colchicine on α_{2B}AR, but not the α_{2A}AR or the α_{2C}AR subtypes, contributes to the growing evidence of differences in trafficking itineraries between these highly similar subtypes in agonist-occupied as well as unoccupied states (von Zastrow et al., 1993; Daunt et al., 1997). Thus, the α_{2B}AR is randomly delivered to apical and basolateral surfaces in polarized MDCKII cells, but selectively retained on the basolateral surface, in contrast to the direct delivery of both the α_{2A}AR and α_{2C}AR subtypes solely to the basolateral surface. After agonist occupancy, the α_{2B}AR, but not the α_{2A}AR or α_{2C}AR subtypes, rapidly and extensively is removed from the cell surface in a number of heterologous cell backgrounds (Eason and Liggett, 1992; Kurose and Lefkowitz, 1994; Schramm and Limbird, 1999). Removal of the third intracellular loop renders the α_{2B}AR incapable of tethering to the plasma membrane, suggesting that the loop of the α_{2B}AR is involved in cell surface membrane anchoring, as has been shown for the α_{2A}AR in MDCKII cells (Keefer et al., 1994; Edwards and Limbird, 1999). The inability of colchicine to mobilize the almost exclusively intracellularly localized α_{2B}AR$\triangle$i3 (Fig. 2) and to increase the density of this mutant receptor suggests that there may be a direct interaction of a microtubule-based cytoskeleton with the third intracellular loop of the α_{2B}AR that contributes to the mechanism by which colchicine increases α_{2B}AR density at the basolateral surface of polarized MDCKII cells. That the effect of colchicine is only observed in polarized cells (Fig. 3) raises the possibility that the protein-protein interactions involved in this phenomenon include proteins that are uniquely synthesized following cell polarization or are that are redistributed to a particular compartment, such as underlying the basolateral surface, on polarization.

The extant literature provides examples where cytoskeletal depolymerization decreases activity of receptors, channels, or enzymes within cells (Hein et al., 1995; Brown et al., 1997; Molitoris, 1997; Cutaia et al., 1998; Schober et al., 1998), as well as evidence that depolymerization can increase activity. For example, actin depolymerization (such as following exposure to cytochalasin D) has been demonstrated to increase Na^+ channel activity in renal epithelial cells (Cantiello et al., 1991) and CFTR-mediated Cl^- current in adenocarcinoma cells (Prat et al., 1995). We did not evaluate the effects of cytochalasin D on α_{2B}AR trafficking and density because this agent leads to loss of polarized expression of a number of endogenous surface proteins in MDCKII cells (Saunders and Limbird, 1997). In contrast, colchicine treatment does not lead to redistribution of the EGFR, a basolateral surface marker protein, or of gp135, an apical marker protein, in MDCKII cells (Saunders and Limbird, 1997).

The present studies raise the possibility that GPCR distribution, density, and/or function may be altered in disease states where the cellular cytoskeleton is altered. Myocardial ischemia is one such example (Hein et al., 1995). Another is hypoxia in pulmonary endothelial cells; the actin filament cytoskeleton is significantly altered after prolonged hypoxic exposure and, as a consequence, its human pulmonary arterial endothelial cell Na^+/H^+ antiport activity is decreased (Cutaia et al., 1998). Ischemia-reperfusion injury also is associated with severe alterations in the cytoskeletal organization of multiple target cells, including polarized renal tubular epithelial cells, where redistribution of a number of polarized membrane transport proteins impairs transepithelial function (Brown et al., 1997; Molitoris, 1997; Schober et al., 1998). In these, and analogous settings, it is reasonable to speculate that disrupted microtubule networks could alter GPCR functional density in general and α_{2B}AR density in particular. If α_{2B}ARs in other polarized cells, such as neurons, are similarly susceptible to microtubule depolymerization, then altered *trans*-synaptic efficiency could occur in a number of settings where disrupted microtubule structure or function is a pathological correlate.

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