The Three $\alpha_2$-Adrenergic Receptor Subtypes Achieve Basolateral Localization in Madin-Darby Canine Kidney II Cells via Different Targeting Mechanisms*

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The present studies examined the localization of the $\alpha_{2A}$- and $\alpha_{2C}$-adrenergic receptor (AR) subtypes in polarized Madin-Darby canine kidney cells (MDCK II) and the mechanisms by which this is achieved. Previously we demonstrated that the $\alpha_{2A}$AR subtype is directly delivered to lateral subdomain of MDCK II cells. Surface biotinylation strategies demonstrated that the $\alpha_{2B}$AR, like the $\alpha_{2A}$AR, achieves 85–90% basolateral localization at steady-state. However, in contrast to the $\alpha_{2A}$AR, this polarization occurs after initial random insertion of the $\alpha_{2B}$AR into both apical and basolateral surfaces followed by selective retention on the lateral subdomain (t½ on the apical surface is 15–30 min; t½ on the basolateral surface is 8–10 h). The $\alpha_{2C}$AR also is enriched on the basolateral surface at steady-state and, like the $\alpha_{2A}$AR, is directly delivered there. Morphological evaluation of the epitope-tagged $\alpha_{2A}$AR, $\alpha_{2B}$AR, and $\alpha_{2C}$AR subtypes by laser confocal microscopy not only corroborated the biochemically-defined basolateral localization of all three $\alpha$AR subtypes but also revealed that the $\alpha_{2C}$AR uniquely exists in an intracellular compartment(s) as well. Immunofluorescence due to intracellular $\alpha_{2C}$AR partial overlaps that due to calnexin, a marker for endoplasmic reticulum, as well as that due to mannosedase II, a marker for the trans-Golgi network. Taken together, the present findings demonstrate that the $\alpha_{2A}$AR, $\alpha_{2B}$AR, and $\alpha_{2C}$AR subtypes, which possess highly homologous structures and ultimately achieve similar polarization to the lateral surface of MDCK II cells, nonetheless manifest distinct trafficking itineraries.

$\alpha_{2}$-Adrenergic receptors ($\alpha_2$ARs) belong to a superfamily of G-protein-coupled receptors that have seven predicted transmembrane spanning regions. The three $\alpha_2$AR subtypes, called $\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$AR, all couple to the G-proteins of the G1 and G$_s$ class and mediate a variety of physiological responses via pertussis toxin-sensitive signal transduction pathways, including inhibition of adenyl cyclase, activation of receptor-operated K$^+$ channels, and suppression of voltage-gated Ca$^{2+}$ channels (1).

The $\alpha_2$AR has been demonstrated in the kidney of several mammalian species (2, 3). Although these receptors are concentrated in the proximal tubular segment of the nephron, they also are found in the glomerulus, thin descending limb of Henle’s loop and cortical collecting duct. The physiological function of the $\alpha_2$AR in the proximal tubule is to increase Na$^+$ reabsorption and proton secretion via the modulation of Na$^+$/$H^+$ exchange (2, 4). The precise physiological role for each of the $\alpha_2$AR subtypes is not yet defined.

The expression of multiple $\alpha_2$AR subtypes in the kidney (5) and the effects of adrenergic agents on renal physiology encouraged us to explore one determinant of receptor-mediated function, namely the precise localization of the receptor molecules in the plasma membrane of renal epithelial cells. Renal epithelial cells are polarized both morphologically and functionally into at least two distinct compartments: apical and basolateral. Both radioligand binding studies and renal microperfusion experiments are consistent with the interpretation that $\alpha_2$ARs are present primarily in the basolateral membrane domain of renal epithelial cells in vivo (6, 7). How this localization is achieved for the $\alpha_2$AR, or for any G-protein-coupled receptor, is only beginning to be revealed.

Recent studies from our laboratory have examined the targeting and retention of wild-type and epitope-tagged $\alpha_2$AR in cultured Madin-Darby canine kidney (MDCK II) cells, a model system that achieves morphological and functional polarity following culture on Transwell filters (8). Surface biotinylation strategies demonstrated that 85–90% of the wild-type $\alpha_2$AR population is localized on the basolateral surface of MDCK II cells, in a manner analogous to the localization of this receptor in vivo. Furthermore, immunoelectron detection of the $\alpha_2$AR, based on the recognition of an artificial epitope tag introduced by mutagenesis into the amino terminus of the receptor, has revealed that the $\alpha_2$AR is not randomly distributed in the basolateral surface but is highly enriched on the lateral sub-domain of polarized MDCK II cells. This localization is achieved by direct delivery to this surface, based on findings from metabolic labeling studies. Extensive mutational analysis of the $\alpha_2$AR to reveal structural regions that confer direct $\alpha_2$AR delivery to the basolateral surface suggests that basolateral targeting of $\alpha_2$AR does not rely on amino-terminal glycosylation, carboxyl-terminal acylation, nor amino acid sequences within the third cytoplasmic loop and the carboxyl-terminal tail (9). These findings are consistent with the interpretation that sequences in or near the lipid bilayer are involved in the delivery of the $\alpha_2$AR to the basolateral surface. In contrast, the deletion of the predicted third cytoplasmic loop of the $\alpha_2$AR significantly decreases its half-life on the epithelial cell surface, suggesting that this structural region of $\alpha_2$AR

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† The abbreviations used are: $\alpha_2$AR, adrenergic receptor; MDCK, Madin-Darby canine kidney; [125I]Rau-AzPEC, 17γ-hydroxy-2β-yohimb-16β-(N-(4-azido-3-$\beta$)-iodo-phenethyl)-carboxamide; HA; hemagglutinin; PCR, polymerase chain reaction; bp, base pair(s); PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; WT, wild-type; MOPS, 4-morpholinepropanesulfonic acid; FITC, fluorescein isothiocyanate.
may participate in mechanisms that stabilize the receptor on the lateral subdomain.

The existence of three subtypes of α2-AR that possess regions of structural similarity and diversity provides natural reagents to further understand the structural regions of G-protein-coupled receptors that confer information for targeting and stabilization in polarized cells. The three α2A-AR subtypes (α2A/AR, α2B/AR, and α2C/AR) can be distinguished not only by differences in pharmacological specificity but also by differences in their primary amino acid sequences. Although sequences in the seven transmembrane-spanning domains are highly conserved among the α2A/AR subtypes, the large third cytoplasmic loop and the amino and carboxyl termini of the α2A/AR subtypes demonstrate significant sequence dissimilarity (10–13). In addition, the post-translational modification of these three receptors is quite different. For example, the α2A/AR has sequences (10, 11) which confer amino-terminal glycosylation (14) and carboxy-terminal acylation (15). In contrast, the α2B/AR is not glycosylated (13, 14, 16), but its carboxyl terminus does contain the sequences appropriate for acylation, whereas the α2C/AR does not contain acylation signals but does possess glycosylation signals in the amino terminus (12).

The structural differences among the α2A/AR subtypes led us to explore the possibility of a different targeting mechanism for each subtype, possibly resulting in differential receptor subtype localization. The present findings demonstrate that although the α2A/AR and α2C/AR subtypes ultimately achieve basolateral localization in steady-state, they do so by different molecular mechanisms. Thus, the α2A/AR shares the direct basolateral delivery pathway characteristic of the α2A/AR, but it is not exclusively localized on the cell surface at steady-state. The α2B/AR, which achieves almost complete basolateral localization at steady-state, does so following apparent random delivery to both surfaces and prolonged retention on the basolateral surface.

**EXPERIMENTAL PROCEDURES**

**Materials**

The α2B/AR (RNG) and α2C/AR (RG10) DNAs were kindly provided by Drs. Kevin Lynch (University of Virginia) and Stephen Lanier (University of South Carolina, respectively). The protein A-purified 12CA5 monoclonal antibody was obtained from the Berkeley Antibody Co. (BABCO), Affi-Gel 10 and Affi-Gel 15 were purchased from Bio-Rad, and the anti-mannosidase II polyclonal antibody (17) was a kind gift from Drs. Marilyn Farquhar (University of California in San Diego) and Kelley Mooreen (University of Georgia). The anti-calmodulin monoclonal antibody (18) was purchased from StressGen Biotechnologies Corp. [3H]Rauwolscine (8 Ci/mmol), [35S]dATP, and [125I]methoxyinulin (125.6 mCi/g) were from Du Pont NEN. The precursor 17α-hydroxy-20α-yohimb-16β-(N-4-aminophenethyl)-carboxamide (Rau-AzPAC) was a kind gift from Dr. Lanier and the radiodinated azido derivative, [3H]Rau-AzPAC, was synthesized in our laboratory as described by Lanier et al. (19). Phenodamine was a gift from CIBA Pharmaceutical Co. Bition-LC-Hydradze, sulfona-NHS-bitin, and streptavidin-agarose were purchased from Pierce. Protein A-agarose was obtained from Vector Laboratories. Dulbecco’s modified Eagle’s medium was prepared by the Cell Care Culture facility supported by the Diabetes Research and Training Center at Vanderbilt University Medical Center. Fetal calf serum was purchased from Sigma. Transwell plates (0.4 μm pore size) were obtained from Costar. Sep-Pak columns were from Millipore Corp. The Sequencing Kit was obtained from U. S. Biochemical Corp. All other chemicals utilized were reagent grade.

**Construction of Epitope-tagged α2A/AR and α2C/AR Receptor Structures**

The α2A/AR and α2C/ARs were epitope-tagged at either the amino or carboxyl terminus. The hemagglutinin (HA) tag was introducted into the amino termini of the α2A/ARs (termed TAG-α2A/AR and TAG-α2C/AR) by site-directed mutagenesis in plBueScript II vector after initial isolation of single-stranded DNA. The hemagglutinin epitope TAG is encoded by the first 9 of the 11 added amino acids (YPYDVPSDYLA). Mutants containing the TAG sequence were confirmed by deoxy DNA sequencing using T7 DNA polymerase and [α-35S]dATP. Appropriate mutant structures were then subcloned into the polylinker region of pCMV4 mammalian expression vector (8). The present DNA sequence was confirmed again.

The introduction of the epitope tag into the carboxyl termini of the α2A/AR and α2C/ARs (termed α2A/AR-TAG and α2C/AR-TAG) was achieved using polymerase chain reaction (PCR)-based mutagenesis. The α2A/AR 5′-oligonucleotide (24-mer) was 5′-GGTACTGAAAGACGTTCGGAAC. The α2A/AR 3′-oligonucleotide containing the hemagglutinin TAG sequence, the stop codon, and an EcoRV restriction site (82-mer with 24 bases annealing) was 5′-CTAGGATATCATCACGAGACGATCCGATGAGAACGTCTCCTG-3′. The 82-mer oligonucleotides were purified on a 9% PAGE, the appropriate bands were excised, eluted, and passed over C18 Sep-Pak columns. The 24-mer oligonucleotides were purified on C18 Sep-Pak columns. The PCR reactions were done at high denaturing temperature (98°C) in the presence of 5% dimethyl sulfoxide following a “hot start” in the absence of DNA polymerase at 90°C for 30 min. Thermal stable VENT™ DNA polymerase was required for these PCR reactions due to a high GC content of the DNA sequences encoding the predicted third cytoplasmic loop of the α2A/AR subtypes. In case of each α2-subtype, double-stranded DNA template (5 ng) was used with the respective oligonucleotides at a final concentration of 0.5 μM. The incubation also contained 0.25 mm dNTPs, 1× VENT™ DNA polymerase buffer, and 1 unit of VENT™ DNA polymerase in a final volume of 300 μl with a top layer of mineral oil. The PCR conditions were as follows: denaturing at 98°C for 1 min, annealing at 68°C for 1 min, extension at 72°C for 2 min for 35 cycles, followed by a 10-min extension at 72°C and storage at 4°C. Obtained PCR products were purified on agarose gels and digested with the appropriate restriction enzymes; α2A/AR-TAG PCR product (167 bp) was cut with EcoRV and BamH1. α2A/AR-TAG PCR product (744 bp) was digested with Smal and Mlu1. Obtained α2A/AR-TAG (99 bp) and α2C/AR-TAG (740 bp) fragments were purified on agarose gels and subcloned into the pCMV4-α2A/AR plasmid (in place of the EcoRV-BsaI fragment encoding wild type sequence) and pCMV4-α2C/AR expression plasmid (instead of the wild type Smal-Mlu1 fragment), respectively.

The sequence of all of these mutant constructs was confirmed by double-stranded deoxy DNA sequencing using T7 DNA polymerase and {α-35S}dATP. Transient transfection of COS M6 cells (100-mm dish plated at 1× 10⁶ cells/dish) with 10 μg of each expression plasmid utilizing the DEAE-dextran method (9) was employed to assess the level of receptor expression using [3H]yohimbine binding as well as to examine the detectability of the epitope TAG by immunocytochemistry prior to utilization of the DNA to develop permanent transformants.

**Creation of Permanent Clonal MDCK II Cell Lines**

Permanent clonal cell lines were developed using the CaPO4 precipitation method, as described previously (8). Briefly, 20 μg of the expression plasmid were co-transfected with 2 μg of pRSVneo into 1× 10⁶ MDCK II cells. Colonies expressing the neomycin resistance gene were selected with the neomycin analog G418 (500 active μg/ml), isolated, and screened for the expression of α2A/AR by measuring the binding of the α2A/AR antagonist [3H]Rauwolscine and the α2A/AR agonist p-([125I]iodolocadine.

**Cell Cultures**

Madin-Darby canine kidney (MDCK II) cells, kindly provided by Dr. Enrique Rodriguez-Boulan (Cornell University), were grown in Dulbeccos modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO₂. MDCK II cells were plated on polycarbonate membrane filters (Transwell) at a density of 1× 10⁶ cells/24.5-mm Transwell and maintained in culture for 7–8 days, changing medium every day, at which time the cells achieved a morphologically and functionally polarized phenotype (8). In the present studies, we examined multiple MDCK II clonal cell lines as summarized in Table I.
**Targeting and Localization of α2-Adrenergic Receptor Subtypes**

**Leak Assay**

Leak assays were performed to determine whether the MDCK II cells developed tight junctions, and served as an indication of their integrity as a monolayer on Transwell filters. [3H]Methoxynoradrenaline was added to the growth medium in the apical compartment and the leak of this radioligand from the apical to basolateral compartment was assessed after a 1-h incubation at 37°C. A typical [3H]Methoxynoradrenaline leak ranged from 1% to 2%. Transwells exhibiting leaks greater than 2% were excluded from study.

**Receptor Localization at Steady-state**

a) Biochemical Approach Utilizing the Surface Biotinylation Strategy—The distribution of the α2-AR on the apical versus the basolateral membrane domain in MDCK II permanent clonal cell lines can be quantitated using surface biotinylation strategies as described previously (8, 9). Briefly, glycoproteins (which include the α2A-AR and α2C-AR) on either the apical or the basolateral surface of MDCK II donor grown on Transwell cell culture system were covalently modified with biotin-LC-hydrazide, while primary amines of the non-glycosylated α2B-AR were covalently labeled with sulfo-NHS-biotin. Following membrane preparation, α2-ARs were identified by covalent modification with the photoactivatable α2AR-selective ligand [125I]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 α2-adrenergic antagonist. The photoaffinity-labeled receptor was then extracted with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 0.5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) and streptavidin-agarose chromatography was employed to isolate the previously biotinylated molecules. The fraction of the biotinylated α2-AR present on the apical versus the basolateral membrane domain was determined SDS-PAGE, autoradiography, and, and counting of radiolabeled bands corresponding to the migration of the α2B-AR, α2C-AR, and α2D-AR.

b) Morphological Approach Employing Immunocytochemistry—Clonal cell lines grown on polycarbonate filters were excised from the Transwell support, rinsed with phosphate-buffered saline (PBS), and fixed for 30 min at 22°C (room temperature) with 4% (v/v) paraformaldehyde in PBS (α2B-AR and α2C-AR) or 15 min with 2% (v/v) paraformaldehyde in PBS (α2A-AR). Cells were rinsed twice with PBS, incubated in 50 mM NH₄Cl solution in PBS for 15 min to quench the fixative, and then permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. Potential sites for nonspecific antibody binding were blocked by a 15-min incubation with 1% BSA, 0.2% Triton X-100, 0.04% sodium azide in PBS at room temperature. The primary antibody, affinity-purified (see below) 12CA5 mouse monoclonal antibody directed against the hemagglutinin epitope, was diluted 1:25 in 1% BSA, 0.2% Triton X-100, 0.04% sodium azide in PBS and incubated with the sample for 1 h at room temperature. This was followed by four 15-min washes with 0.2% Triton X-100 in PBS and a 1-h incubation with a secondary Cy3-conjugated donkey anti-mouse IgG (1:100 dilution in 1% BSA, 0.2% Triton X-100, 0.04% sodium azide in PBS). The cells were washed as before with PBS containing 0.2% Triton X-100 and mounted on glass microscope slides with Aqua Polymount. The results were analyzed either on a Leitz Fluorescent Microscope using a 40 × oil immersion lens or on a Zeiss laser confocal microscope.

Immunological co-localization studies were done essentially as described above, although expanded by the additional application of another primary and secondary antibody. Incubation with 1:400 dilution of the anti-calnexin rabbit polyclonal antibody (used as a marker for the endoplasmic reticulum (18)), or a 1:1000 dilution of anti-mannosidase II rabbit polyclonal antibody (used to detect trans-Golgi network (17)) in 1% BSA, 0.2% Triton X-100, 0.04% sodium azide followed the initial detection of the α2B-AR with 12CA5 antibody and FITC (1:60 dilution) or Cy3 (1:100 dilution)-conjugated donkey anti-mouse IgG. The anti-calnexin or anti-mannosidase II primary antibodies were incubated with the sample for 1 h at room temperature, followed by four 15-min washes with 0.2% Triton X-100 in PBS and a 1-h incubation with a secondary donkey anti-rabbit FITC (1:60 dilution) or Cy3 (1:200 dilution)-conjugated IgG in 1% BSA, 0.2% Triton X-100, 0.04% sodium azide in PBS. The cells were then washed four times for 15 min with PBS, 0.2% Triton X-100, mounted, and analyzed as described above.

Since the initial immunoanalysis of α2A-AR localization in MDCK II cells revealed a significantly lower level of fluorescent signal as compared to the α2B-AR, we generated additional α2A-AR and α2C-AR DNA constructs and clonal cell lines. We were concerned that the epitope tag of the TAG-α2A-AR was not easily accessible to 12CA5 antibody because this subtype has an unusually high alanine content (17 alanine residues in its amino-terminal region, potentially leading to the formation of helices. Consequently, we deleted the 3'-untranslated regions (280 bp in TAG-α2A-AR and approximately 500 bp in TAG-α2C-AR) in order to...
Targeting and Localization of α2-Adrenergic Receptor Subtypes

FIG. 2. Immunological localization of the three α2AR subtypes in polarized MDCK II cells using 12CA5 antibody. MDCK II clones expressing the epitope-tagged α2AR subtypes (TAG-α2A AR 3, TAG-α2B AR 122, and TAG-α2C AR 11) were polarized on the Transwell system, fixed, and immunostained as described under "Experimental Procedures." The localization of the hemagglutinin epitope tag determined by immunocytochemical strategies described in detail under "Experimental Procedures" was analyzed on a Zeiss laser confocal microscope. Localization of the epitope-tagged α2AR, α2B AR, and α2C AR in the XY plane is presented in the lower panel of each image set. Z scans shown in the upper panel of each image set show a laser-sectioned side view of MDCK II cells expressing each of the three receptor subtypes. The yellow line across each XY plane represents the exact site where the cells were sectioned from top to bottom with the laser beam to create the Z scan.

RESULTS AND DISCUSSION

In the present studies, we examined whether the three subtypes of α2AR are differentially localized in MDCK II cells and whether the modes of receptor delivery to the cell surface or their half-lives on the membrane after surface delivery differ among the three subtypes.
Targeting and Localization of α2-Adrenergic Receptor Subtypes

All Three α2AR Subtypes Achieve Basolateral Localization in MDCK II Cells at Steady-state—Steady-state localization of each α2AR subtype was determined using surface biotinylation strategies in multiple permanent clonal cell lines grown in Transwell culture. The α2Rs were identified using photoaffinity labeling with [125I]Rau-AzPEC. The specificity of labeling was documented by comparing the profile of radiolabeled bands on SDS-PAGE incubated with [125I]Rau-AzPEC in the absence or presence of a non-α2-adrenergic antagonist, phenotamine. As shown in Fig. 1, the α2CAAR (Fig. 1A) and α2C-TAG AR (Fig. 1C) subtypes migrate just above the 66-kDa molecular mass marker, consistent with the glycosylation of these 442 (α2CAAR) and 461 (α2C-TAG AR) amino acid receptors. In contrast, the α2CAR subtype migrates at approximately 45 kDa on SDS-PAGE, as expected for this non-glycosylated 453 amino acid receptor subtype (14, 16). Quantitation of the fraction of each α2AR subtype that is biotinylated on the basolateral versus apical surface indicates that 91% of the WT-α2AR, 89% of the TAG-α2AR, 97% of the WT-α2CAR, 85% of the TAG-α2CAR, 95% of the WT-α2C-TAG AR, and 92% of the TAG-α2C-TAG AR are localized on the basolateral surface at steady-state. Furthermore, the fraction of α2C-TAG AR or α2CAR enriched on the basolateral surface was not altered quantitatively if the epitope tag was introduced into the amino carboxyl terminus of the receptor. Comparable amounts of the radiolabeled receptor are detected in detergent-solubilized extracts obtained from Transwell cultures biotinylated on either the apical or basolateral surface prior to streptavidin-agarose chromatography (Ref. 8; data not shown). Thus, the overwhelming greater basolateral signal detected following streptavidin-agarose chromatography of the extracts from biotinylated cells is a true reflection of α2AR enrichment on the basolateral surface of MDCK II cells. The finding that introduction of the epitope tag did not alter the apparent localization of any of the α2AR subtypes meant that these epitope-tagged structures could be exploited in subsequent immunocytochemical and metabolic labeling studies and yet faithfully represent the fate of the wild-type α2AR subtype structure.

Since surface biotinylation strategies only reveal the fraction of the receptor population at either the apical or basolateral surface, we also employed immunocytochemical methods to evaluate the surface as well as possible intracellular localization of each of the α2AR subtypes within MDCK II cells. MDCK II clones were grown in Transwell culture and immunostained using the Affi-Gel-purified 12CA5 antibody directed against the epitope tag (see “Experimental Procedures”). The surface staining pattern of the three α2AR subtypes corroborates the basolateral localization revealed using surface biotinylation strategies. For the α2CAAR and α2CAR, the immunofluorescence was detected exclusively on the lateral subdomain of MDCK II cells (Fig. 2). The α2CAR, however, is localized not only on the lateral subdomain, but also in intracellular compartments. Our findings in MDCK II cells resemble previous reports of both surface and intracellular localization of the α2CAR when expressed in COS-7 and HEK-293 renal cell lines (21). This bi-modal surface and intracellular localization of the α2CAR, unique among the α2AR subtypes, must represent a property of the α2CAR structure per se, since the same 12CA5 antibody preparation was used to immunologically localize all three subtypes of the α2AR. Furthermore, as mentioned above, introduction of the epitope tag into the amino- versus the carboxyl-terminal domains of the α2CAR did not alter its steady-state distribution, assessed using either biochemical or morphological strategies. Finally, we also examined several independent clonal cell lines (see Table I), and found that the relative distribution of α2AR for all subtypes was unmodified over the range of 2 to 25 pmol of α2AR/mg of protein. Thus, we are confident that the receptor distribution shown in Fig. 2 represents properties of the structures of the varying α2AR subtypes.

Comparison of the Localization of Intracellular α2CAR with Calnexin and Mannosidase II—In order to explore the cellular compartment(s) where the intracellularly-localized α2CAR...
might be expressed, we compared the immunological localization of the $\alpha_2$AR with that of calnexin (18), a marker for the endoplasmic reticulum, and mannosidase II, a marker for the trans-Golgi network (17). Fig. 3 compares the localization of $\alpha_2$AR (Panels A and D) with mannosidase II (Panels B and E). Panel C and F in Fig. 3 examine the co-localization (yellow color) of $\alpha_2$AR (green color due to the FITC-conjugated secondary antibody) and mannosidase II (red color due to the Cy3-conjugated secondary antibody). Laser sectioning in the Z plane is most informative in revealing the extent, or lack thereof, of $\alpha_2$AR co-localization with markers for the endoplasmic reticulum and trans-Golgi network. As shown in Fig. 3C, the staining for the intracellular $\alpha_2$AR completely overlaps that observed for mannosidase II, whereas in other cross-sections (e.g. Fig. 3F) the immunofluorescence of the intracellular $\alpha_2$AR expands apically from the trans-Golgi network, which is defined by mannosidase II staining. Similarly, the profile of the intracellular $\alpha_2$AR immunofluorescence partially overlaps that for calnexin, but also occupies cellular space oriented apically from endoplasmic reticulum (Fig. 3I), as defined by calnexin staining. Taken together, the findings in Fig. 3 (C, F, and I) suggest either that the intracellular $\alpha_2$AR is distributed throughout the endoplasmic reticulum and trans-Golgi network, or is localized to a unique subcellular compartment that is characterized by an immunofluorescence profile that partially overlaps both calnexin and mannosidase II staining.

The $\alpha_2$AR, Like $\alpha_2$AR, Is Delivered Directly to the Basolateral Membrane of MDCK II Cells. While the $\alpha_2$AR Is Initially Randomly Inserted into Both Apical and Basolateral Surfaces—Our next goal was to determine if all three subtypes of $\alpha_2$AR are directly delivered to the basolateral surface of MDCK II cells, where they predominantly reside at steady-state, or alternatively, if $\alpha_2$AR subtypes achieve this steady-state localization via distinct mechanisms. Delivery of the $\alpha_2$AR subtypes was examined by metabolic labeling of nascent receptors, followed by biotinylation on the apical or basolateral cell surface, immunosolubilization using the anti-epitope tag antibody, and quantitation of the fraction of $\alpha_2$AR biotinylated on a particular surface following streptavidin-agarose chromatography.

Metabolic labeling studies require the epitope-tagged receptor as a means to identify and isolate the $\alpha_2$AR among the other radiolabeled proteins. Thus, for these studies we were restricted to using clonal cell lines permanently expressing the epitope-tagged versions of $\alpha_2$AR subtypes to study receptor delivery to the cell surface. Fortunately, all of our steady-state data comparing the localization of photoaffinity-labeled $\alpha_2$AR indicate that introduction of the epitope tag does not influence the localization of any of the $\alpha_2$AR subtypes (cf. Fig. 1). It was of interest, however, that we were able to immunosolubilize the $\alpha_2$AR subtype only when the epitope tag was inserted into the carboxyl terminus; perhaps the alanine-rich sequence in the amino terminus of the $\alpha_2$AR rendered the tag inaccessible to the 12CA5 antibody when inserted in the amino terminus.

Fig. 4 examines the delivery of all three $\alpha_2$AR subtypes in MDCK II cells. In all cases, we are confident that the radiolabeled band identified as $\alpha_{2AAR}$, $\alpha_{2BAR}$, or $\alpha_{2CAR}$ represents the particular subtype under study, because of its comigration with photoaffinity-labeled receptor and its absence when comparable metabolic labeling studies are performed in parental MDCK II cells lacking expression of any of these subtypes. The band migrating just above the 66-kDa molecular mass marker on SDS-PAGE is interpreted to represent the $\alpha_{2AAR}$ (Fig. 4A) and $\alpha_{2CAR}$ (Fig. 4C) subtype. Newly synthesized $\alpha_{2CAR}$ subtype (Fig. 4C), like the $\alpha_{2AAR}$ (Fig. 4A), appear virtually exclusively on the basolateral surface after each labeling time point tested. These data are consistent with the interpretation that both the $\alpha_{2AAR}$ and $\alpha_{2CAR}$ subtypes are delivered directly to the basolateral surface. In contrast, the $\alpha_{2BAR}$ shows a fundamentally different targeting pattern when compared to the $\alpha_{2AAR}$ and $\alpha_{2CAR}$ subtypes. As shown in Fig. 4B, the 45-kDa $\alpha_{2BAR}$ is initially randomly inserted into both apical and basolateral surfaces, where it is first detectable 30 min after initiation of metabolic labeling. This random expression on both the apical and basolateral surfaces also is repeatedly observed 45 min after pulse labeling of this receptor subtype. However, 60 min after the initiation of metabolic labeling, the majority of the $\alpha_{2BAR}$AR is found on the basolateral membrane. The data shown in Fig. 4B are from one experiment repeated 6 times with comparable outcome. The $\alpha_{2BAR}$AR subtype delivery was assessed using receptor structures where the epitope tag was introduced either into the amino or carboxyl terminus, and the indistinguishable results obtained for either epitope-tagged structure support that position of epitope tag does not influence the apparent random delivery of the $\alpha_{2BAR}$AR in MDCK II cells.

![Fig. 4](http://www.jbc.org/Downloaded from)
The α2B-AR subtype is selectively retained on the basolateral surface—One possible scenario that could explain the initial uniform distribution of the α2B-AR on apical and basolateral surfaces followed by enrichment on the basolateral surface would involve selective retention of the α2B-AR in the basolateral, but not in the apical, domain. To determine if the α2B-AR was selectively retained on the basolateral surface, we directly examined the half-life of the α2B-AR on both the apical and basolateral surfaces of MDCK II cells. Cells grown in Transwell culture were metabolically labeled with [35S]cysteine/methionine, and then chased in medium supplemented with 1 mM methionine and 1 mM cysteine for the indicated time periods. After completion of the chase period, cells were biotinylated on either the apical (A) or the basolateral (B) surface with NHS-biotin. Subsequently, the α2B-AR was isolated via sequential protein A-agarose and streptavidin-agarose chromatography, and resolved on SDS-PAGE. The upper panels of A and B show representative autoradiograms. Upon excision and counting gel bands that correspond to the α2B-AR, the percentage of radioactivity present in each receptor band was plotted as a function of time (radioactivity present in t₀ (time = 0) band was ascribed as 100%). The means ± S.E. of radioactivity present in α2B-AR corresponding gel bands averaged from n = 7 (A) and n = 3 (B) are shown in the lower panels of A and B. The calculated half-life of the α2B-AR on the apical surface was 15–30 min, whereas the calculated half-life of the α2B-AR on the basolateral surface was 8–10 h.

Fig. 5. Differential retention of the α2B-AR subtype on the apical versus the basolateral surface of MDCK II cells. MDCK II cells permanently expressing the α2B-TAG-AR clone 42 (A) or TAG-α2B-AR clone 122 (B) were metabolically labeled with 150 μCi of [35S]cysteine/methionine in 150 μl of medium at 37 °C, 5% CO₂ for 30 (A) or 60 (B) min, then chased in medium supplemented with 1 mM methionine and 1 mM cysteine for the indicated time periods. After completion of the chase period, cells were biotinylated on either the apical (A) or the basolateral (B) surface with NHS-biotin. Subsequently, the α2B-AR was isolated via sequential protein A-agarose and streptavidin-agarose chromatography, and resolved on SDS-PAGE. The upper panels of A and B show representative autoradiograms. Upon excision and counting gel bands that correspond to the α2B-AR, the percentage of radioactivity present in each receptor band was plotted as a function of time (radioactivity present in t₀ (time = 0) band was ascribed as 100%). The means ± S.E. of radioactivity present in α2B-AR corresponding gel bands averaged from n = 7 (A) and n = 3 (B) are shown in the lower panels of A and B. The calculated half-life of the α2B-AR on the apical surface was 15–30 min, whereas the calculated half-life of the α2B-AR on the basolateral surface was 8–10 h.

Fig. 6. Retention of the three α2-AR subtypes on the basolateral surface. MDCK II permanent clonal cell lines expressing the epitope-tagged α2-AR subtypes used in representative autoradiograms shown above included α2A-TAG-AR 52 (A), TAG-α2B-AR 122 (B), and α2C-TAG-AR 28 (C). Polarized MDCK II cells grown on three or four 24-mm Transwell filters (depending on the clonal receptor expression) for 7 days were metabolically labeled (pulsed) with 150 μCi of [35S]cysteine/methionine in 150 μl of medium for 60 min at 37 °C, 5% CO₂, and then incubated in medium supplemented with 1 mM methionine, 1 mM cysteine for various periods of time (“chase period”) at 37 °C. The 2-h chase period was designated as t₀ (time = 0) based on our previous observations of time required for the nascent α2-AR to arrive at the basolateral surface. In addition to the t₀, α2-ARs were also isolated following 6- and 24-h chase periods, using biotin surface labeling strategy coupled with receptor immunosolubilation and streptavidin-agarose chromatography, as described under “Experimental Procedures.” The upper panel of A, B, and C provides autoradiograms of 7–20% gradient SDS-PAGE from a representative experiment; the lower panels show plots of the means ± S.E. of radioactivity in each band averaged from three experiments. Receptor bands were excised from autoradiograms according to the position of each α2-AR subtype and counted in a β-counter with 10 ml of scintillation liquid. The percentage of radioactivity present in each receptor band was plotted on a semi-log scale as a function of time defining radioactivity at t₀ as 100%.
surface or, alternatively, is internalized and degraded. However, the differential stability of $\alpha_2$BAR on the apical versus the basolateral membrane explains the observation that, at steady-state, the $\alpha_2$BAR is localized almost exclusively on the basolateral surface. Furthermore, this finding suggests that each membrane domain has a unique mechanism for protein retention and, moreover, that the apical retention mechanism for the $\alpha_2$AR is less efficient.

Fig. 6 demonstrates that the $\alpha_2$AR, $\alpha_2$AR, and $\alpha_2$CAR have comparable half-lives on the basolateral surface. Previous data from our laboratory suggest, at least for the $\alpha_2$AR, that receptor retention involves protein-protein interactions involving endofacial domains of the receptor, since deletion of the third cytoplasmic loop of the $\alpha_2$AR measurably accelerates receptor turnover on the basolateral surface (9). The similar half-life of the $\alpha_2$AR, $\alpha_2$BAR, and $\alpha_2$CAR subtypes on the basolateral surface suggests that similar tethering mechanisms may exist for all three structures despite the fact that the endofacial third loop sequences of these three $\alpha_2$AR subtypes are quite distinct. Perhaps these $\alpha_2$AR subtypes, when they achieve their three-dimensional structure, project similar surfaces to endofacial proteins that stabilize $\alpha_2$ARs on the surface. More rapid turnover of the $\alpha_2$AR on the apical domain suggests that if such tethering proteins do exist, they may be absent or exist in a reduced density underneath the apical surface, resulting in a more rapid $\alpha_2$BAR turnover on the apical, when compared with the basolateral, domain.

**SUMMARY**

Fig. 7 provides a schematic diagram of the different trafficking itineraries observed for the three $\alpha_2$AR subtypes in these studies. As described previously (8), the $\alpha_2$AR subtype is delivered directly to the basolateral membrane and at steady-state virtually all $\alpha_2$AR is present on the surface. The $\alpha_2$AR also is directly delivered to the basolateral surface, but at steady-state is distributed between cell surface and one or more intracellular compartments. Finally, the $\alpha_2$CAR subtype is initially delivered randomly to both cell surfaces but then is preferentially retained on the basolateral membrane. Although all three $\alpha_2$AR subtypes have been detected in renal epithelia of varying species (2, 3, 5), the functional consequences of these differing receptor itineraries in renal epithelial cells revealed by the present studies or the implications for targeting and retention of these $\alpha_2$AR subtypes in other polarized cells, such as neurons, remain to be established.

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