Receptors Coupled to Pertussis Toxin-sensitive G-proteins Traffic to Opposite Surfaces in Madin-Darby Canine Kidney Cells

A1 ADENOSINE RECEPTORS ACHIEVE APICAL AND α2A ADRENERGIC RECEPTORS ACHIEVE BASOLATERAL LOCALIZATION*

(Received for publication, October 10, 1995)

Christine Saunders‡, Jeffrey R. Keefer, Amy P. Kennedy, Jack N. Wells, and Lee E. Limbird§

From the Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37209

*This work was supported by National Institutes of Health Grants DK 43879 (to L. E. L.) and GM 21220-18 (to J. N. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Recipient of a postdoctoral fellowship in pharmacology-morphology from the Pharmaceutical Research and Manufacturers of America Foundation.

§To whom correspondence should be addressed: Dept. of Pharmacology, Vanderbilt University Medical Center, MRB 464, Nashville, TN 37209-6600. Tel.: 615-343-3538.

The α2A adrenergic receptor (α2AAR) previously was shown to be directly delivered to and retained on the lateral subdomain of renal epithelial cells. The present studies demonstrate that, in contrast, wild-type and epitope-tagged canine A1 adenosine receptors (A1AdoR) are apically enriched (65–83%) in Madin-Darby canine kidney (MDCKII) and porcine renal epithelial (LLC-PKI) cells, based on surface biotinylation strategies detecting photoaffinity-labeled A1AdoR. Confocal microscopy corroborated the apical enrichment of the epitope-tagged A1AdoR. Metabolic labeling studies revealed that this steady-state polarization is achieved by direct delivery to both the apical (60–75%) and basolateral surface. Growth of A1AdoR expressing cells as monolayers was achieved only following Transwell culture in the presence of A1AdoR antagonists, which decreased cell proliferation. This preferential apical but detectable basolateral localization of A1AdoR provides a molecular understanding of published reports that functional responses can be elicited following apical as well as basolateral delivery of adenosine agonists in varying renal preparations. These findings also suggest that receptor chimeras derived from the Gia1 and Gi2 protein-coupled α2AAR and A1AdoR will be informative in revealing structural features critical for basolateral versus apical targeting.


to Opposite Surfaces in Madin-Darby Canine Kidney Cells

It is important to understand the molecular properties that govern polarized expression of epithelial cell proteins, as this polarity is an intrinsic part of the vectorial functioning of these cells. The coordinated cellular functions mediated by endogenous and exogenous ligands depend on the availability of appropriate receptors at the particular surface domains to which the ligand has access. Our laboratory is interested in elucidating the mechanisms and structural regions within G-protein-coupled receptors responsible for polarized expression of these regulatory molecules in renal epithelial cells. We have demonstrated previously that the α2AAR is predominantly localized (>85%) to the basolateral surface of Madin-Darby canine kidney (MDCKII)3 cells, a polarized model system for renal epithelia that accurately reflects α2AAR localization in vivo (1). Immunolocalization studies revealed that the α2AAR is enriched in the lateral subdomain of the basolateral surface, and metabolic labeling studies indicated that the α2AAR is directly targeted to the basolateral domain (1).

The present studies characterize the localization and delivery of another seven transmembrane-spanning G-protein-coupled receptor, the A1 adenosine receptor (A1AdoR), in polarized renal epithelial cells. Interestingly, this receptor is enriched on the apical surface in both MDCKII and porcine renal LLC-PKI cells. In the kidney, adenosine, present in both blood and urine (2), acts as a paracrine regulator of renal blood pressure, renin secretion, and renal excretion. The A1AdoR, like the α2AAR, regulates cellular processes by interacting with G-proteins in the Gia1 and Gia2 family. Although some have explored the localization of the adenosine receptors by means of functional studies (3–7), there is no evidence of the localization of these receptors using direct biochemical and morphological strategies. In an attempt to study the localization of the A1AdoR in renal epithelial cells, we have created permanent clonal cell lines of MDCKII and LLC-PKI cells expressing either a wild-type or epitope-tagged A1AdoR. The apical versus basolateral distribution of the A1AdoR in polarized renal epithelial cells was determined biochemically and morphologically, and the general mechanism by which this predominantly apical localization is achieved was explored.

EXPERIMENTAL PROCEDURES

Materials—8-Cyclopentyl-1,3-di-(2,3[3H])propylxanthine ([3H]DPCPX, 109 Ci/mmol), ExpreSS™S™ protein labeling mixture (1200 Ci/mmol), [3H]methoxyinulin (125.6 mCi/g), and [3H]S9ATP (1389 Ci/mmol) were purchased from DuPont NEN. The A1AdoR antagonist, 1,3-dipropyl-8-(4-sulfophenyl)xanthine or DPPSX, was prepared according to Daly et al. (8) Biotin hydrazide and streptavidin-agarose were purchased from Pierce. The protein A-purified 12CA5 monoclonal antibody was purchased from the Berkeley Antibody Co. The gp125 and EGF receptor monoclonal antibodies were generously donated by Dr. Peter Dempsey (Vanderbilt University). Cy-3-conjugated donkey antimouse IgG was purchased from Jackson Immunoresearch.

Cell Culture and Functional Confirmation of Intact Monolayers—Madin-Darby canine kidney (MDCKII) cells were obtained from Enrique Rodriguez-Boulan (Cornell University, New York, NY) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (complete Dulbecco's modified Eagle's medium) at 37 °C, 5% CO2. LLC-PKI clone 4 cells were obtained from Anne E. Rubega-

The abbreviations used are: MDCKII, Madin-Darby canine kidney; α2AAR, α2A adrenergic receptor; A1AdoR, A1 adenosine receptor; HA, hemagglutinin; WT, wild-type; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; [3H]DPCPX, 8-cyclopentyl-1,3-di-(2,3[3H])propylxanthine; DPPSX, 1,3-dipropyl-8-(4-sulfophenyl)xanthine.
Male in the laboratory of Carolyn Slanyan (Yale University, New Haven, CT). The parental cells and the permanent cell lines were maintained in a minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 units/ml penicillin G, and 100 μg/ml streptomycin (minimum essential complete medium) in a 5% CO₂, 37°C incubator.

In this study, using transfected MDCKII and LLC-PK1 cells were seeded at a density of 1×10⁶ cells/24.5 mm polycarbonate membrane filter (Transwell chambers, 0.4-μm pore size, Costar, Cambridge, MA), and cultured for 5–8 days with medium changes every day. Where stated, cells were grown in the presence of 60 μM theophylline and 100 μM DMSO. Prior to use in each experiment, the monolayer was assayed for [3H]methoxyinulin and Krebs medium by monitoring the leak of [3H]methoxyinulin from the apical compartment to the basolateral compartment by sampling and counting the basolateral medium in a β-counter (Packard Tricarb) after a 1-h incubation at 37°C. Chambers with leaks greater than 3%/h were discarded.

Construction of the A₁ and TAG-A₁ Adenosine Receptor—RCD7 (canine thyroid adenosine A₁ receptor cDNA) cloned into the pBluescript SK + plasmid at the EcoRI site was obtained from the laboratory of J. E. Dumont and G. Vassart, Université Libre de Bruxelles, Brussels.

A.2.08-kilobase KpnI-XbaI fragment containing the A₁AdoR full-length coding region, plus 53 bases of 5’-noncoding and 997 bases of 3’-noncoding sequence, was excised and cloned into the KpnI-XbaI sites of the mammalian expression vector pCMV4. To create the epitope-tagged A₁AdoR, a truncated 1–9 amino acid (YPYDVPDYALVPR) to the extracellular amino terminus of the canine A₁AdoR immediately after the initiator methionine (9). The first 9 amino acids constitute a hemagglutinin (HA) epitope (10) recognized by the commercially available monoclonal antibody 12CA5 (Berkley Antibody Co.), while the last 4 amino acids were added as a spacer arm. Positive mutations were verified using denaturing DNA sequencing (Sequenase kit, U. S. Biochemical Corp.) utilizing T7 DNA polymerase with [α-35S]dATP. Once verified, the insert encoding the epitope tag was subcloned into the pCMV4-A₁AdoR plasmid (11), and reverified with sequencing. After the retransfer from pCMV4-A₁AdoR and pCMV4-TAG-A₁AdoR constructs were transiently transfected into COSM6 cells. Membranes from these transfectants were used for binding of the A₁AdoR antagonist, [³H]DPCPX (see below). Binding of [³H]DPCPX to the epitope-tagged A₁AdoR was indistinguishable from binding to the wild-type A₁AdoR.

Development of Permanent Transformants of LLC-PK1 and MDCKII Cells—the pCMV4-A₁AdoR (20 μg) or pCMV4-TAG-A₁AdoR adenosine receptor (20 μg) was co-transfected with 2 μg of pRSVneo into 1×10⁶ MDCKII cells using polyethyleneimine (Promega) precipitation method (12), except that 2 days following transfection, 500 μg of active G418/ml was added either directly to the original plate of transfected cells or to plates containing different dilutions of the transfected cells. G418-resistant colonies were screened for A₁AdoR expression by assaying the binding of the A₁AdoR antagonist [³H]DPCPX (13). Specific [³H]DPCPX binding was defined as that binding not competed for by 1 mM A₁AdoR antagonist, [³H]DPCPX (13). Specific [³H]DPCPX binding was defined as that binding not competed for by 1 mM A₁AdoR antagonist, [³H]DPCPX (13). Specific [³H]DPCPX binding was defined as that binding not competed for by 1 mM theophylline. Untransfected MDCKII cells displayed no detectable specific binding of [³H]DPCPX.

A.₁ Adenosine Receptor Binding Assay—MDCKII and LLC-PK1 cell membrane particulate preparations were prepared essentially as described (11) with the following modifications. The lysis buffer was 15 mM Tris-HCl, 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. The final pellet was resuspended in 900 μl of membrane binding buffer (50 mM Tris, 10 mM MgCl₂, 6H₂O, pH 7.4). The assay of [³H]DPCPX binding was performed in 12×75-mm polypropylene tubes containing 3 nM [³H]DPCPX (di-lutated with 100 μM theophylline), 50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4) in the absence (total binding) or presence (nonspecific binding) of 1 mM theophylline. Incubations were for 45 min at 25°C. Reactions were terminated by the addition of 3.5-ml ice-cold membrane binding buffer and filtration through Whatman GF/B glass microfiber filters, which were prewetted with 0.3% polyethyleneimine in membrane binding buffer at 4°C for 20 min. Upon termination of the binding assay, the filters were dried at 60°C for 20 min and were counted in 5 ml of NEN-NI93 liquid scintillation fluid.

Steady state localization of the A₁AdoR, Adenosine Receptors: Biotin Surface Labeling Strategy—The method for quantitating the apical versus basolateral distribution of the wild-type and epitope-tagged A₁AdoR in polarized MDCKII cells was biotinylation of the apical versus basolateral surface of cells grown in Transwell culture, photoaffinity labeling of the functional A₁AdoR in harvested membranes, detergent extraction, and isolation of biotinylated receptors via streptavidin-agarose chromatography. The studies were performed essentially as described previously (1). Briefly, MDCKII or LLC-PK1 cells grown in Transwell culture were washed three times for 5 min in PBS containing 0.5 mM CaCl₂ and 1 mM MgCl₂ (PBS/CM) and 1 mM theophylline. Following sequential treatment with sodium periodate and biotin-LC-hydrazide, cells were harvested into PBS containing 1 mM theophylline, 100 μM DMSO, and 0.1 mM phenylmethylsulfonyl fluoride and membranes were prepared as described above. A small sample of the membrane preparation was removed and assayed for [³H]DPCPX binding. To photoaffinity label the A₁AdoR in these membranes, aliquots containing 25,000–50,000 cpm of specific [³H]DPCPX binding were incubated with 2 nM A₁AdoR antagonist, ³H-azido-BW-A844U (13), for 45 min in membrane binding buffer at room temperature in the dark. Non-specific photoaffinity labeling was assayed in a separate incubation in the presence of 1 mM theophylline, added 15 min before ³H-azido-BW-A844U. Photocorporation was initiated by irradiation of the membrane preparations with ultraviolet light at 254 nm (Rayonet miniphotomicrochemical reactor) for 3 min. The photoaffinity-labeled membranes, derived from Transwell cultures that had been biotinylated on either the apical or the basolateral side, were collected by centrifugation, resuspended in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) and prepared for streptavidin-agarose chromatography as described previously (1). The eluates were then subjected to SDS-PAGE and autoradiography, and the area of the gel corresponding to the A₁AdoR signal was excised and counted on a Betaxon 5000 γ-counter. The amount of ³H-azido-BW-A844U-labeled A₁AdoR on the apical versus basolateral surface.

Metabolic Labeling/ Biotinylation Strategy for Determining Surface Delivery of the TAG-A₁ Adenosine Receptor—The amount of newly synthesized A₁AdoR delivered to the apical versus basolateral surface was quantified by biotinylation one surface or the other of metabolically labeled MDCKII cells in Transwell culture and subsequently isolating radiolabeled receptor on the biotinylated surface through sequential immunoprecipitation and streptavidin-agarose chromatography. These procedures were performed essentially as described (1), except that membranes were prepared in adenosine receptor membrane binding buffer, as described above.

Immunolocalization of A₁ Adenosine Receptor—Primary and secondary antibodies were optimized using cells plated on coverslips before evaluating cells polarized by culture in Transwells. For immunostaining of MDCKII cells on coverslips, cells were plated at confluence on glass coverslips and cultured for 2–3 days prior to staining. Cells were fixed and stained with a 1:50 dilution of 12CA5 primary antibody as described (1). Upon rinsing the cells after the incubation of the primary antibody, a 2:200 dilution of the secondary Cy3-conjugated donkey anti-mouse IgG was added to the cells in PBS containing 2% bovine serum albumin and incubated for 1 h at room temperature in the dark. The cells then were mounted on glass slides with Aqua-Poly Mount (Polysciences Inc., Warrington, PA).

For immunostaining of cells grown in Transwell culture, cultures were grown for 4–6 days and assessed for binding of [³H]methoxyinulin leak; wells with leaks greater than 3% were discarded. The cells on the polycarbonate filter were fixed and stained using the same protocol as those grown on the coverslips, except that the filter was excised from the Transwell support prior to the first antibody incubation. Samples were visualized by confocal microscopy on a Zeiss Axiovert 135 Micro Systems LSM (Germany). The samples were first visualized in the xy plane, and then in the xz plane; the images were downloaded onto a Silicon Graphics Iris Indigo workstation for analysis using Showcase software.

RESULTS

The Wild-type and the Epitope-tagged A₁ Adenosine Receptor Are Enriched on the Apical Membrane Domain of MDCKII Cells—To examine the trafficking of the A₁- and TAG-A₁ AdoR in canine renal epithelial cells, clones of MDCKII cells permanently expressing wild-type and epitope-tagged receptors were made. The cell lines characterized in most detail in this study expressed 17.2 pmol of wild-type A₁AdoR/mg protein (clonal line A₁AdoR#10) and 36.9 pmol of TAG-A₁AdoR/mg of total membrane protein (clonal line TAG-A₁AdoR#17). Two other independent untagged cell lines of lower densities also were evaluated; the findings with all cell lines evaluated were comparable.

To determine the steady-state localization of the A₁AdoR, we
employed surface biotinylation and subsequent photoaffinity labeling. The A1AdoR extracted from membrane preparations and eluted from streptavidin-agarose was evaluated using SDS-PAGE. The A1AdoR migrated on SDS-PAGE with an apparent mass of 43–45 kDa; this photo labeled species was not detected when the incubation with 125I-azido-BW-A844U was carried out in the presence of the A1AdoR antagonist, theophylline. The A1AdoR was enriched on the apical surface in cell lines expressing both the wild-type (65–68%) and the TAG-A1AdoR (75–83%) structures (Fig. 1A). These data indicate that the presence of the HA-epitope did not significantly alter the apical enrichment of the A1AdoR at steady state.

A Similar Apical Localization Is Detected for the A1 Adenosine Receptor in LLC-PKI Cells—Because there are examples of the TAG-A1AdoR. The specificity of identification of the

2 M. Caplan, personal communication.

![Fig. 1.](image1.png)

**Fig. 1.** The wild-type and epitope-tagged A1AdoR are enriched on the apical membrane of MDCKII cells at steady state. A, the MDCKII clonal cell lines expressing either wild-type A1AdoR (A1AdoR#10) or epitope-tagged A1AdoR (TAG-A1AdoR#7) were grown in Transwell culture and treated with biotin hydrazide to selectively label either the apical or the basolateral membrane surface. In the experiments shown here, three Transwell cultures were pooled for each surface determination. The A1AdoR was identified with photoaffinity labeling using 125I-azido-BW-A844U as described under “Experimental Procedures”; non-receptor labeling was assessed in the absence of the adenosine receptor antagonist, theophylline. The autoradiogram was exposed for 7 days to Kodak X-Omat film between two Quanta III screens at –70 °C. Gel slices corresponding to the position of the A1AdoR signal on autoradiograms were excised and counted in a γ-counter. The findings shown are representative of two separate experiments. B, confocal microscopy images of the MDCKII clonal cell line A1AdoR#10 or TAG-A1AdoR#17 cultured on Transwell filters, fixed, and incubated, in the presence of 0.1% Triton X-100, with a 1/50 dilution of 12CA5 monoclonal antibody directed agains the hemagglutinin epitope as described under “Experimental Procedures.” The x-y z scan shows the cells oriented with the apical surface facing up toward the eye-piece. The yellow line represents the section of cells through which the z scan was made. The z scan at the top of the photograph represents a longitudinal cut through the cells, where the horizontal signal represents the apical (top) surface of the cells. (See Fig. 5, B and D, for comparison with pattern of endogenous protein expression characteristic of apical versus basolateral localization.) Experiments in both A and B were performed on cells grown in A1AdoR antagonists (100 μM DPSPX and 50 μM theophylline) to permit cell growth as monolayers (see “Results”).
HA epitope with the 12CA5 antibody is further revealed by comparison of localization of the TAG-A₁AdoR with the basolaterally targeted TAG-α₂AR structures in subsequent figures (cf. Figs. 5 and 6).

Growth of A₁AdoR-expressing MDCKII Cells as a Polarized Monolayer Requires the Presence of Adenosine Receptor Antagonists—Upon growing the A₁AdoR-expressing clones in culture, we observed that the growth pattern of the adenosine receptor-transfected MDCKII cells looked undifferentiated from other MDCKII cell clones, in particular, growth in patches and in multilayers. We noted that when cells were plated and grown in the presence of two adenosine receptor antagonists, DPSPX (a hydrophilic, highly selective A₂ receptor antagonist) and theophylline (a non-subtype selective A₁/A₂ receptor antagonist), the cells were prevented from forming multilayers in culture. To evaluate more directly the effect of A₁AdoR antagonists on MDCKII cell growth, subconfluent A₁AdoR-transfected and untransfected MDCKII cells were grown in the absence and presence of 60 μM theophylline, a nonspecific adenosine receptor antagonist, for 2 days, at which point the cells were harvested and counted. Theophylline significantly inhibited the growth rate of the A₁AdoR-transfected cells, but not untransfected MDCKII cells (Fig. 3A). Furthermore, cell number achieved over time in the A₁AdoR-transfected cells was greater compared to the untransfected cells when both were plated at the same density and grown in the absence of theophylline (2.77 ± 0.24 A₁AdoR-transfected cells/dish versus 1.92 ± 0.29 untransfected cells/dish; p = 0.058, where n = 5 for each group). These findings suggest that A₁AdoR, either via theophylline-sensitive, agonist-independent receptor activation of MDCKII cells or in response to ambient adenosine, a proliferative agent in some cells (16, 17), evokes cell growth. To assess whether or not stimulation of growth of MDCKII cells is a characteristic response to other G₁/Go-coupled receptors in these cells, we examined the effect of the α₂AR agonist, UK-14304, on cells in which the α₂AR had been introduced by transfection (1); the α₂AR agonist had no significant effect on cell growth (Fig. 3B). These findings indicate that introduction of the A₁AdoR, but not of the α₂AR, changes the growth properties of the MDCKII cells, suggesting (albeit not to the exclusion of other explanations) that the A₁AdoR and the α₂AR are localized in distinct compartments containing different G₁/Go-coupled effector systems. Although the precise mechanisms by which adenosine receptors mediate proliferation of MDCKII cells was not explored in these studies, our studies did establish that growth of Transwell cultures in the presence of adenosine receptor antagonists prevented multilayer cellular accumulation. (This interpretation is corroborated by the immunocytochemical data in Fig. 4B, described below.)

Because characterization of receptor polarization by biochemical means requires a non-permeable monolayer, we examined whether the proliferation of cells expressing adenosine receptors grown in the absence of adenosine receptor antagonists might have contributed to the variable (65–85%) apical localization of A₁AdoR reported in preliminary studies from our laboratory (18). We thus compared the relative apical localization of A₁AdoR in Transwell cultures grown in the absence and presence of the combined adenosine receptor antagonists, theophylline (60 μM) and DPSPX (100 μM) using both biotinylation (Fig. 4A) and immunocytochemical (Fig. 4B) strategies. It was apparent from the immunolocalization experiments that in most clonal cell lines, the cells grew in a multilayer in the absence of adenosine receptor antagonists (Fig. 4B). In some cases, as in Fig. 4A, the percent apical polarization estimated from streptavidin-agarose recovery of biotinylated and photo-labeled A₁AdoR increased notably when growth in Transwell culture occurred in the presence of antagonists, and in this example was 56% in the absence and 75% in the presence of antagonists. Since cell growth in multiple layers would confound analysis of receptor localization by biochemical strategies, these two receptor antagonists were included in all subsequent experiments of all cell lines expressing the A₁AdoR.

Immunolocalization of Epitope-tagged A₁AdoR, α₂AR, and...
α2AAR in MDCKII Cells—To confirm the apical localization of the epitope-tagged A1AdoR, we compared the localization of the A1AdoR with endogenous MDCKII markers for the apical and basolateral surfaces, and with the α2AAR, already characterized in terms of its lateral targeting following expression in MDCKII cells (1). The apical staining observed for TAG-A1AdoR#17 (Fig. 5A) was consistent with the immunostaining pattern of an endogenous MDCKII cell protein, gp135 (19), that serves as a marker for the apical surface in MDCKII cells (21). The confocal image of the TAG-A1AdoR#17 in MDCKII cells grown on Transwell filters (Fig. 5B) shows a lateral staining pattern indistinguishable from the image of the known, endogenous EGF receptor, which serves as a marker for the basolateral surface in MDCKII cells (20). The appearance of areas with no cells in the x-y scan of the A1AdoR-stained cells is attributed to the various heights of the A1AdoR-expressing cells in the presence (Fig. 6A) or absence (Fig. 6B) of Triton X-100. The apical staining is somewhat fainter when the procedure is performed in the absence of Triton X-100, perhaps because the detergent facilitates access of the 12CA5 antibody to the amino-terminal epitope on the A1AdoR even on the extracellular surface. To test our ability to detect the HA epitope when expressed intracellularly, we also examined MDCKII cells expressing the α2AAR receptor subtype, which previously has been demonstrated to be localized on the surface as well as in an intracellular compartment in MDCKII cells, as well as COS and human embryonic kidney cells (21). The ability to readily identify the intracellular compartment containing the TAG-α2AAR structure required incubation of the fixed Transwells with primary antibody in the presence of Triton X-100 (Fig. 6C), as the detection of this intracellular compartment was not clearly visible in the absence of Triton X-100 (Fig. 6D). These control studies indicate that if a substantial fraction of the TAG-A1AdoR were enriched in an intracellular compartment, incubation with the 12CA5 primary antibody in the presence of Triton X-100 should have detected it.

The A1 Adenosine Receptor Is Delivered Directly to the Apical and Basolateral Surfaces of MDCKII Cells—To investigate the mechanism by which apical enrichment is achieved, the delivery of the A1AdoR to the apical versus the basolateral surface was determined using metabolic labeling in combination with the surface biotinylation (Fig. 7). Newly synthesized, metabolically labeled A1AdoR was first detectable after 30 min of pulse labeling. At this time, metabolically labeled receptor was detectable on both the apical and basolateral surfaces, but appeared to be preferentially delivered to the apical surface (63% apical, 37% basolateral). Over all the time points examined (30, 45, 60, 90, and 120 min), the apical to basolateral ratio remained relatively constant, consistent with the interpretation that the amount of labeled A1AdoR detected at each surface is due to direct delivery to these surfaces (Fig. 7A). We are confident that the labeled protein, migrating slightly above

---

**Fig. 4.** Growth in the presence of adenosine receptor antagonists fosters MDCKII cell growth as a monolayer in A1AdoR-expressing cell lines. Cells were grown either in the absence or presence of the adenosine receptor antagonists, theophylline (60 μM) and DPSPX (100 μM). A, biochemical characterization of A1AdoR localization (clonal cell line TA1AdoR#17) cultured on Transwell filters, fixed, and stained as described under “Experimental Procedures.” Both the x-y and z scan reveal that cells are piled on top of each other in the absence of the antagonists, whereas growth in the presence of antagonists results in a cell monolayer detected in both the x-y and z scans.

**Fig. 5.** Comparison of the A1AdoR localization with the basolateral α2AAR and endogenous protein markers of apical and lateral surfaces. The confocal image of the TAG-A1AdoR#17 in MDCKII cells grown on Transwell filters in the presence of A1AdoR antagonists (A) reveals apical staining over the entire cell surface in the x-y scan and the horizontal line of red in the z scan. This is indistinguishable from the confocal image of the endogenous protein, gp135, which serves as a marker for the apical surface in MDCKII cells (B). The confocal image of the TAG-α2AAR (also tagged (1) with the same HA epitope used to identify the A1AdoR) in MDCKII cells grown on Transwell filters (C) shows a lateral staining pattern indistinguishable from the image of the known, endogenous EGF receptor, which serves as a marker for the basolateral surface in MDCKII cells (D).

---

3 M. Wozniak and L. E. Limbird, unpublished observations.
the 43-kDa molecular size marker, is the A1AdoR, since it comigrates with the photolabeling- and with the photoaffinity-labeled A1AdoR (A1AdoR#10) and because no radioactive band is visible at this molecular weight when MDCKII cells expressing wild-type A1AdoR (A1AdoR#10) are metabolically labeled for 60 min and processed exactly as MDCKII cells expressing A1AdoR#7 (Fig. 7). Since the steady-state localization of a receptor is influenced by both the initial delivery to a particular surface and the eventual retention to that specific surface, we examined the half-life of the A1AdoR (A1AdoR#7) on both the apical and basolateral surface. The kinetics of the removal of the receptor from the apical plasma membrane were not significantly different from those of the basolateral membrane (Fig. 8). The calculated half-life on the apical surface was about 9-13 h and on the basolateral surface about 11-12 h. Thus, the apical enrichment observed at steady state cannot be attributed to differential retention of the A1AdoR on the apical compared to the basolateral surface, but appears to result from preferential delivery to the apical surface.

DISCUSSION

Endogenous compounds or drugs must first be recognized by the appropriate receptor or, in the case of synthetic compounds, receptor subtype to exert their desired effect. Multiple mechanisms likely contribute to specificity in signal transduction by endogenous agonists: the existence of numerous receptor subtypes that couple to distinct signal transduction pathways; coupling of receptors to distinct effector systems in various tissues; and receptor localization to discrete microdomains on the cell surface, restricting the G-proteins, effectors, and other molecules with which the receptor interacts. The importance of receptor localization in signal transduction is inferred by the number of pathophysiologic states that result from mislocalized receptors (22, 23). For example, one form of retinitis pigmentosa results from intracellular trapping of the G-protein-coupled receptor for light, rhodopsin. It therefore is essential to understand the mechanisms that govern the trafficking of receptors and signal transducing proteins in order to both gain insights into their role in receptor-mediated signal transduction events under physiologic conditions and to probe for potential culprits in pathophysiologic states.

We previously have demonstrated that the α2AAR is localized basolaterally in renal epithelial cells (1). One strategy that could reveal the structural regions of the α2AAR, and perhaps all G-protein-coupled receptors, necessary for conferring basolateral localization is to make receptor chimeras with other seven-transmembrane-spanning, G-protein-coupled receptors that achieve opposite localization in polarized cells. We chose the A1AdoR as a potential candidate since adenosine is available in sufficient concentrations (2) to activate adenosine receptors on the apical surface of renal cells in vivo due to the existence of cavedar 5'-nucleotidase in that domain (24). Also, in vivo studies, Franco et al. (25) found that an A1AdoR antagonist, when administered lumenally, participated in the tubuloglomerular feedback mechanism. However, it should be noted that responses to basolaterally introduced adenosine receptor agonists also have been demonstrated in renal epithelial cells in vitro, e.g., A1AdoR-accelerated phosphatidylinositol turnover and inhibition of forskolin-stimulated cAMP production following basolateral but not apical administration of adenosine (3). Similarly, basolaterally localized A1AdoR have been suggested by addition of A3 analogues to the basolateral compartment (7) or to the basolateral surface of perfused inner medullary collecting duct tubules (5).

Our studies are the first to characterize the localization of the A1AdoR based on its structure, using biochemical and morphological detection strategies, rather than relying on receptor-mediated functions to infer receptor localization. Our studies demonstrate that the A1AdoR is preferentially localized to the apical surface of MDCKII and LLC-PK1 cells in multiple independent clonal cell lines. The apical enrichment of the A1AdoR at steady state is mirrored by the preferential delivery of A1AdoR to the apical versus basolateral surface. Since the A1AdoR has a similar half-life on the apical and the basolateral surfaces after its delivery, our findings are consistent with the interpretation that the A1AdoR localization is achieved by direct delivery to the apical versus basolateral surfaces. It may be that both the apically and basolaterally localized receptor populations elicit activities important for polarized function, albeit by coupling to different effector mechanisms. Our own observations on proliferation of MDCKII cells expressing the A1AdoR (Fig. 3) are consistent with this interpretation; both the A1AdoR and the α2AAR elicit responses via Gz/Gi-coupled GTP-binding proteins, but only introduction of the A1AdoR led to an increase in cell number in polarized MDCKII cells. Whether or not the effects of theophylline to slow cell doubling are due to agonist-independent effects of the receptor or result from endogenously produced adenosine acting at the heterologous A1AdoR has not been determined. Nevertheless, inclusion of A1AdoR antagonists was necessary to obtain a monolayer of cells required for performing polarization studies.

The finding that the A1AdoR is delivered to both the apical and basolateral surfaces, but preferentially to the apical surface, distinguishes this receptor from other G-protein-coupled receptors characterized to date. The α2AAR is delivered exclusively to the basolateral surface (1); in contrast, the α2BAR...
Fig. 7. **The A\textsubscript{1}AdoR is preferentially delivered to the apical membrane domain of MDCKII cells, consistent with its steady-state enrichment on that surface.** A. MDCKII cells expressing epitope-tagged A\textsubscript{1}AdoR (TAG-A\textsubscript{1}AdoR\#7) grown in Transwell culture in the presence of A\textsubscript{1}Ado antagonists were metabolically labeled with 1 \(\mu\)Ci/\(\mu\)l \[^{35}\text{S}\]Met/[^{35}\text{S}\]Cys protein labeling mix (150 \(\mu\)l) for the indicated times and then harvested and processed using sequential immunopurification and streptavidin-agarose chromatography as described under "Experimental Procedures." The autoradiograph shown is for an SDS-PAGE gel excised and counted for 7 days to Kodak X-Omat film between two Quanta III screens at \(-70^\circ\text{C}\). Gel slices corresponding to the position of the A\textsubscript{1}AdoR signal on autoradiograms were excised and counted in a 10 ml of NEN-963 scintillation fluid in a \(\beta\)-scintillation counter. Localization on the apical domain was not significantly different over the range of time points examined; the findings from multiple individual experiments evaluating varying time points (in percent apical enrichment) were as follows: 30 min (n = 2) mean = 61, range = 59–63; 45 min (n = 3) mean = 67.3, range = 62–76; 60 min (n = 6) mean = 62.3, range = 56–66; 90 min (n = 2) mean = 57.5 range = 55–60; 120 min (n = 1) is 62% apical enrichment. B, cells expressing wild-type A\textsubscript{1}AdoR (clone 10) were metabolically labeled for 60 min and then treated as in panel A. The autoradiograph for metabolically labeled wild-type receptor was exposed for 7 days with Kodak X-Omat film at \(-70^\circ\text{C}\). Migration of the photoaffinity-labeled A\textsubscript{1}AdoR was included in the experiment to demonstrate the migration of the A\textsubscript{1}AdoR for comparison with the metabolically labeled, epitope-tagged protein isolated by sequential protein A and streptavidin-agarose chromatography. In all gels where TAG-A\textsubscript{1}AdoR were analyzed, there were visible \[^{35}\text{S}\]Met/Cys-labeled bands migrating at about 55, 67, 80, and 94 kDa; these presumably correspond to receptor aggregates, since they also were seen when the A\textsubscript{1}AdoR was identified by photoaffinity labeling. No \[^{35}\text{S}\]Met/Cys signal was detected at any molecular weight when cells expressing the wild-type receptor were analyzed.  

Acknowledgments—We thank Carol Ann Bonner for technical assistance in the maintenance of all cell lines and development of several of the A\textsubscript{1}AdoR-expressing cell lines evaluated. We are grateful to Dr. Magdalena Woźniak for providing the TAG-\(\alpha\text{AR}\)-expressing MDCKII cell line used in the immunolocalization comparison with TAG-\(\alpha\text{AR}\) in Fig. 7. We thank Dr. Peter Dempsey for the gift of the gp135 and EGF receptor monoclonal antibodies. We are also grateful to Dr. Tom Jetton for constant and patient assistance with the use of the confocal microscope and discussions concerning immunocytochemical techniques. J. R. K. acknowledges Dr. Michael Welch (University of Iowa) for his suggestion to test the A\textsubscript{1}AdoR for possible apical localization in MDCKII cells. We are very appreciative to the members of the Limbird laboratory for helpful discussions and assistance during these experiments. Finally, we thank Dr. Enrique Rodriguez-Boulan for providing us with the parental MDCKII cells.

Fig. 8. **The A\textsubscript{1}AdoR has a comparable surface half-life on both the apical and basolateral surface of MDCKII cells.** MDCKII cells expressing TAG-A\textsubscript{1}AdoR\#7 and grown in Transwell culture in the presence of A\textsubscript{1}Ado antagonists were metabolically labeled as in Fig. 7A with 1 \(\mu\)Ci/\(\mu\)l \[^{35}\text{S}\]Met/[^{35}\text{S}\]Cys for a 60-min "pulse" and rapidly rinsed in PBS before the "chase" phase was initiated by addition of complete Dulbecco’s modified Eagle’s medium supplemented with adenine. At the designated time points the cells were rinsed in PBS, and the "chase" phase was initiated by addition of 1 mM methionine, 1 mM cysteine, and A\textsubscript{1}AdoR antagonists. For monitoring of half-life of the A\textsubscript{1}AdoR, the time = 0 corresponds to a 6-h chase period, since after this time point no increase in metabolic labeling signal occurs at the surface. A\textsubscript{1}AdoR present on the apical surface of labeled cells at each time point was calculated by surface biotinylation, followed by sequential immunoprecipitation and streptavidin-agarose chromatography of cell extracts, as described under "Experimental Procedures." Each data point represents the streptavidin-agarose eluate derived from three Transwells per time point per surface evaluated. Gel slices corresponding to the position of the A\textsubscript{1}AdoR signal on autoradiograms were excised and counted in a 10 ml of NEN-963 scintillation fluid and counted in a Packard-TriCarb liquid scintillation counter on the \(\beta\)-channel. The data also were analyzed by scanning the gel on a phosphorimager, where the results were not significantly different from those obtained from cutting and counting the gel slices. The data (in h) are as follows: the calculated half-life ranged from 9–13 h (n = 2) on the apical surface and 11–12 h (n = 3) on the basolateral surface.

Achieves steady-state basolateral localization in MDCKII cells via random delivery and selective retention on the basolateral surface. In studies not characterized as rigorously as those presented here, we observed that the M2 and M3 muscarinic receptors are localized to both the apical and basolateral surfaces of MDCKII cells as revealed by confocal microscopy, but preferentially on the basolateral surface. Thus, all of the G-protein-coupled receptors studied to date, the A\textsubscript{1}AdoR is the only structure preferentially delivered to the apical surface that appears to be retained at that surface without redistribution to the lateral subdomain. Chimeric structures between A\textsubscript{1}AdoR and the \(\alpha\text{AR}\) might therefore permit identification of structural regions in G-protein-coupled receptors that confer basolateral versus apical targeting.

**REFERENCES**

1. Kefer, J. R., and Limbird, L. E. (1993) J. Biol. Chem. 268, 11340–11347
2. Barfuss, D. W., McCann, W. P., and Katoli, R. E. (1992) Kidney Int. 41, 1143–1149
3. Le Févé, D. G., McCoy, D. E., and Spielman, W. S. (1992) Am. J. Physiol. 263, C729–C735
4. Pratt, A., Clancy, G., and Welsh, M. J. (1988) Am. J. Physiol. 251, C167–C174
5. Edwards, R. M., and Spielman, W. S. (1994) Am. J. Physiol. 266, F791–F796
6. Sinko, J. P., Lazarow, A. M., and Boucher, R. C. (1995) Am. J. Physiol. 268, C425–C433
7. Yagi, C., Katni, G., and Yagi, Y. (1994) Pflügers Arch. 427, 225–232
8. Daly, J. W., Padgett, W., Shamin, M. T., Butts-Land, P., and Waters, J. (1985) J. Med. Chem. 28, 487–492
9. Zolzer, M. J., and Smith, M. (1983) Methods Enzymol. 100, 468–500
10. Wilson, I. A., Nimni, H. L., Houghten, R. A., Charness, A. R., Connolly, M. L., and Lerner, R. A. (1984) Cell 37, 767–778
11. Guyer, C. A., Horstman, D. A., Wilson, A. L., Clark, J. D., Craigie, E. J., and Saunders, J. Wess, L. E. Limbird, unpublished observations.

*C. Saunders, J. Wess, L. E. Limbird, unpublished observations.*
Limbird, L. E. (1990) J. Biol. Chem. 265, 17307–17317
12. O'Brien, R. M., Lucas, P. C., Forest, C. D., Magnuson, M. A., and Granner, D. K. (1990) Science 249, 533–537
13. Linden, J. (1991) FASEB J. 5, 2668–2676
14. Olah, M. E., Ren, H., Ostrowski, J., Jacobson, K. A., and Stiles, G. L. (1992) J. Biol. Chem. 267, 10764–10770
15. Tucker, A. L., Linden, J., Robeva, A. S., D'Angelo, D. D., and Lynch, K. R. (1992) FEBS Lett. 297, 107–111
16. Rozengurt, E. (1982) Exp. Cell Res. 139, 71–78
17. Meininger, C. J., and Granger, H. J. (1990) Am. J. Physiol. 258, H198–H206
18. Keefer, J. R., Saunders, C., Kennedy, A. P., Wells, J. N., and Limbird, L. E. (1995) FASEB J. 9, A1405
19. Ojakian, G. K., and Schwimmer, R. (1988) J. Cell Biol. 107, 2377–2387
20. Dempsey, P. J., and Coffey, R. J. (1994) J. Biol. Chem. 269, 16878–16889
21. von Zastrow, M., Link, R., Daunt, D., Barsh, G., and Kobilka, B. (1993) J. Biol. Chem. 268, 763–766
22. Brown, M. S., and Goldstein, J. L. (1986) Science 232, 34–47
23. Fish, E. M., and Molitoris, B. A. (1994) N. Engl. J. Med. 330, 1580–1588
24. Anderson, R. G. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10909–10913
25. Franco, M., Bell, P. D., and Navar, L. G. (1989) Am. J. Physiol. 257, F231–F236