Regulation of adenylyl cyclase in polarized renal epithelial cells by G protein-coupled receptors

MARK D. OKUSA, LIPING HUANG, AKEMI MOMOSE-HOTOKEZAKA, LONG P. HUYNH, AND AMY J. MANGRUM

Department of Medicine, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Okusa, Mark D., Liping Huang, Akemi Momose-Hotokezaka, Long P. Huynh, and Amy J. Mangrum. Regulation of adenylyl cyclase in polarized renal epithelial cells by G protein-coupled receptors. Am. J. Physiol. 273 (Renal Physiol. 42): F883–F891, 1997.—We employed two guanine nucleotide binding protein (G protein)-coupled receptors known to be targeted to opposite domains in renal epithelial cells to test the hypothesis that the polarized receptor expression of receptors regulates the activity of the receptor’s effector molecule, adenylyl cyclase. We used LLC-PK1 cells stably transfected with cDNA encoding the α2-adrenergic receptor (α2-AR) or A1-adenosine receptor (A1-AdR). Immunohistochemistry and Western blot analysis confirmed the basolateral and apical expression of α2-ARs and A1-AdRs, respectively. Atenolol and cycloheximide abolished forskolin-stimulated (10 µM) cAMP accumulation following the addition of forskolin (10 µM) in the presence of 3-isobutyl-1-methylxanthine to apical or basolateral chambers of confluent monolayers. A five- to sixfold increase in cAMP accumulation occurred following apical (or basolateral) stimulation of LLC-PK1 cells expressing apical (or basolateral) receptors in comparison to forskolin stimulation of corresponding domains of untransfected cells. We conclude 1) adenylyl cyclase activity is present at or near the apical and basolateral domains of LLC-PK1 cells, and 2) factors that regulate the polarized expression of inhibitory G protein-coupled receptors may also regulate local adenylyl cyclase activity.

LLC-PK1 cells; adenosine receptors; adrenoceptors; adenosine 3’,5’-cyclic monophosphate; polarity

METHODS

Cell culture. LLC-PK1 cells (American Type Culture Collection, Rockville, MD) were maintained in medium 199 (GIBCO-BRL Laboratories, Grand Island, NY) supplemented with 10% (vol/vol) dialyzed fetal bovine serum (FBS; GIBCO-BRL Laboratories) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (GIBCO-BRL) under a 5% CO2 atmosphere at 37°C. Subculturing was performed every 4–5 days.

For experiments aimed at examining the distribution of receptors and their interaction with adenylyl cyclase, cells were seeded onto polycarbonate membrane permeable filter supports (24.5 mm, 0.45 µm, Transwell chambers; Costar, Cambridge, MA) and grown to confluence. The degree of monolayer confluence was assessed by two independent methods prior to each experiment. Membrane leakage rate of inulin was assessed by adding [3H]inulin (395 mCi/g; Du Pont-NEN, Boston, MA) to the apical medium for 1 h at 37°C. Samples of medium from basolateral and apical chambers were removed, and radioactivity was determined by scintillation counting. Chambers were used only when apical to basolateral leakage rates were less than 2%/h. A second method used to assess the degree of confluence was established by the measurement of resistance across cells, using modified electrodes and an epithelial voltm-ohmmeter (EVOM; World Precision Instruments, Sarasota, FL).

Expression of RNG α2-AR and FLAG A1-AdR in LLC-PK1 cells. RNG α2-AR cDNA previously subcloned into the plasmid RLDN (9) (provided by Dr. Kevin R. Lynch, Department of Pharmacology, University of Virginia) and human FLAG epitope-tagged A1-AdR cDNA previously subcloned into pDT (19) (provided by Dr. Joel Linden, Department of Medicine, University of Virginia) were used to transflect LLC-PK1 cells as previously described (7). The FLAG epitope consists of a 24-nucleotide sequence 5’ to the coding region of the cDNA encoding the human A1-AdR. cDNAs encoding α2-ARs or A1-AdRs were transfected into LLC-PK1 cells in a ratio of 1:1, and cell lysates were assayed for adenylyl cyclase activity. The FLAG epitope, which contains the sequence (in JM) 4N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 276 NaCl, 10 KCl, 3 Na2HPO4·7H2O, and 11 dextrose (pH 7.1) and precipitated by the addition of 1 ml of inhibitory G protein-coupled receptors at the apical membrane of epithelial cells (16, 22), the mechanism by which these receptors couple to adenylyl cyclase is not known. Adenylyl cyclase is thought to be localized to the basolateral domain (24). If this indeed were the case, then it would appear that adenylyl cyclase is spatially segregated from apical receptors to which they couple. The purpose of our study was to examine the influence of polarization of receptor expression on the activity of their effector molecule, adenylyl cyclase. Toward this end we employed A1-adenosine receptors (A1-AdRs), receptors known to be localized to the apical membrane of renal epithelial cells (22), and α2-adrenergic receptors (α2-ARs), receptors known to be expressed at the basolateral membrane of proximal tubule cells (10).

DISTINCT CELL SURFACE proteins at apical and basolateral domains of renal epithelial cells are vital to the transport of ion and water across renal epithelium (4). With cloning of renal epithelial cell transporters, much work has focused on the polarized distribution of these transport proteins. Recently, however, a growing body of literature suggests that receptors are also asymmetrically expressed in renal epithelial cells. Although cell surface receptors in renal epithelial cells are generally regarded as being expressed at the basolateral domain, functional studies have demonstrated their expression at the apical membrane as well. Angiotensin II (23), β2-adrenergic (8), and adenosine receptors (6, 15) are thought to mediate the effects of agonist exposure at the apical domain. Filtered catecholamines or endogenous ligands secreted into the tubular fluid may have distant or local effects by activation of apically expressed receptors.

Although guanine nucleotide binding protein (G protein)-coupled receptors are known to be expressed at both apical and basolateral domains, much less is known regarding the effector molecules to which they couple. For example, despite the demonstration of
0.25 M CaCl₂ and 40 µM chloroquine for 15 min. Trypsinized LLC-PK₁ cells (1 × 10⁶) were pelleted and resuspended in 2 ml of precipitated cDNA suspension and allowed to stand at room temperature for 15 min. Medium was supplemented with 12.5 mM CaCl₂ and 2 mM chloroquine (final concentration), and the suspended cells were seeded onto 100-mm tissue culture dishes and incubated at 37°C, 5% CO₂ for 6 h. Following the incubation period, the medium was aspirated, and cells were subjected to osmotic shock with 5 ml of 20% dimethyl sulfoxide in complete medium 199 for 5 min at 37°C. Cells were washed and grown for 24 h in complete medium 199 before selection in G-418 (0.5 µg/ml) (GIBCO-BRL). Resistant colonies were isolated 2–3 wk after transfection and screened for receptor expression. Receptor densities were determined by using ³H-labeled MK-912 (81.3 Ci/mmol; DuPont-NEN), an α₂-AR-selective agonist, or ³H]-cyclopropanecarbodiimide (DPCPX; 120 Ci/ml, DuPont-NEN), an A₁-AdR-selective antagonist, for radioligand binding assays.

Membrane protein isolation. Cells were grown to confluence on 150-cm² tissue culture plates, harvested in a solution consisting of 250 mM sucrose, 10 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 10 µg/ml each of apotinin, leupeptin, and pepstatin and homogenized. The homogenate was centrifuged at 6,000 × g for 10 min, and the resultant postmitochondrial supernatant was removed and centrifuged at 130,000 × g for 1 h. The supernatant was removed and replaced with 0.25 M sucrose, 10 mM tris(hydroxymethyl)aminomethane, 1.3-dipropylxanthine (DPCPX; 120 Ci/ml, DuPont-NEN), and the biotinylated membrane proteins were recovered by overnight incubation at 4°C with avidin-agarose beads. Bovine serum albumin (200 µg/ml) was added to the bead slurry to block nonspecific binding, and the biotinylated membrane proteins were recovered by washing with PBS-C/M and 10 mM sodium periodate to oxidize biotin to aldehydes. Cells were incubated for 30 min at 4°C with a solution consisting of 0.5 mg/ml biotin LC hydrazide required prior incubation of cells for 30 min at 4°C in PBS-C/M and 10 mM sodium periodate to oxidize glycopolymers to aldehydes. Cells were incubated with gentle agitation for 30 min at 4°C, then washed with medium and PBS-C/M three times. Filters were removed and extracted with 1 ml of ice-cold lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, and 1 mM EDTA). The supernatant was clarified by centrifugation at 14,000 × g for 10 min at 4°C, and the biotinylated membrane proteins were recovered by overnight incubation at 4°C with avidin-agarose beads. Beads were pelleted, washed, resuspended, and saved for Western blot analysis.

Membrane proteins were then incubated for 1 h at room temperature with either an antibody to the α₂B-AR (10) (2.5 µg/ml) or an anti-FLAG monoclonal antibody (27 µg/ml) to detect epitope-tagged A₁-AdRs. Blots were washed, incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h, washed, and reacted with chemiluminescent substrate (ECL detection reagents, Amersham Arlington Heights, IL). Immunoreactivity was detected by exposure of nitrocellulose membranes to X-ray film (Kodak XAR-5).

In some experiments membrane proteins were analyzed following deglycosylation from LLC-PK₁ cells expressing A₁-AdRs, using N-Glycanase (Genzyme, Cambridge, MA). Membrane proteins were denatured by first incubating in a solution containing 0.5% SDS with 50 mM N-acetylglucosamine (final concentrations) and then boiling for 5 min. A 10-µl aliquot of this solution was incubated in 1.25% Nonidet P-40 (final concentration) and 0.3 U of N-Glycanase overnight at 37°C to remove oligosaccharides. The following day, reactions were stopped by the addition of loading buffer, and proteins were subjected to SDS-PAGE and Western blot analysis.

Quantitative assessment of band densities was performed by scanning densitometry using a flatbed scanner (Color OneScanner; Apple, Cupertino, CA) and software analysis program (Scan Analysis; Biosoft, Cambridge, UK).

cAMP assays. Adenosine 3',5'-cyclic monophosphate (cAMP) accumulation was measured under maximal stimulatory conditions following inhibition of phosphodiesterase to assess the activity of adenylyl cyclase at apical and basolateral domains. Untransfected LLC-PK₁ or LLC-PK₁ cells transfected with the α₂B-ARs or A₁-AdRs were grown to confluence on permeable filter supports. Medium was aspirated and cells were incubated for 1 h with fresh medium 199 containing 50 mM N-(2-hydroxyethyl)piperazine-N'-2-hydroxypropane sulfonic acid (HEPES; pH 7.4) in the absence of serum. Plates were chilled to 4°C while drugs were added. Drugs were added to either apical or basolateral chambers, and plates were placed in a water bath and rapidly rewarmed to 37°C. Chambers were incubated with drugs for 10–15 min, after which 0.3 ml of 0.1 N HCl was added to the apical side, and an
aliquot was then removed and frozen until cAMP was assayed. cAMP accumulation was measured by an automated gammaflow radioimmunoassay system (RIA core facility, University of Virginia) as described by Brooker et al. (3).

Adenylyl cyclase activity was determined by the addition of forskolin (10 µM) to either apical or basolateral chamber following the addition of 3-isobutyl-1-methylxanthine (IBMX, 1 mM) for 10–15 min. The dose response characteristics of forskolin on cAMP accumulation in preliminary studies are shown in Fig. 1. On the basis of these results, we chose an intermediate concentration of forskolin (10 µM) that yielded a consistent and measurable response. Figure 2 demonstrates the time course for activation of adenylyl cyclase following forskolin administration. On the basis of these results, cAMP accumulation in subsequent experiments was measured following incubation with forskolin for 10–15 min. Drugs used to assess receptor coupling were N6-cyclopentyladenosine (CPA, 1 µM; Research Biochemical International, Natick, MA), an A1-AdR-selective agonist (14), or UK-14304 (1 µM; Pfizer, Sandwich, Kent, England), an A2-agonist (18). The inhibitory effect of apical addition of CPA on forskolin-stimulated cAMP accumulation was determined. Similarly, we assessed the degree of coupling of A2B-ARs to adenylyl cyclase by determining the inhibitory effect of basolateral addition of UK-14304 (1 µM) on forskolin-stimulated cAMP accumulation.

Our controls in all experiments utilized untransfected cells, because in preliminary experiments control cells and those transfected with the vector alone yielded either comparable results or only minor differences. The apical membrane response to forskolin in the presence of IBMX for untransfected LLC-PK1 cells and LLC-PK1 cells transfected with RLDN was 68.2 ± 9.88 (n = 6) and 119.7 ± 21.07 (n = 6) pmol/mg protein, respectively [P = not significant (NS)]. The basolateral response to forskolin was 42.9 ± 5.34 (n = 6) and 70.6 ± 6.57 (n = 6) pmol/mg protein for untransfected LLC-PK1 cells and cells transfected with RLDN, respectively (P < 0.05).

Data were analyzed by using the unpaired Student’s t-test where appropriate.

RESULTS

A2B-ARs are expressed at the basolateral domain of LLC-PK1 cells. To test the hypothesis that polarization of receptor expression influenced the activity of the receptor’s effector molecule, LLC-PK1 cells were stably transfected with cDNA encoding the rat A2B-AR. Saturation binding studies utilizing [3H]MK-912 demonstrated a receptor density of ~2 pmol/mg protein. No detectable binding was found in untransfected LLC-PK1 cells.

Steady-state immunolocalization studies were performed utilizing a well-characterized affinity-purified polyclonal antibody raised to the third intracellular loop of the A2B-AR (10). As shown in Fig. 3, A2B-ARs were localized primarily to the lateral domains of transfected LLC-PK1 cells. These findings are similar to results described previously (29). No detectable immunoreactivity was observed in untransfected cells (data not shown).

We sought independent biochemical confirmation that LLC-PK1 cells expressed A2B-ARs at the basolateral domain by performing Western blot analysis of biotinylated surface membrane proteins. LLC-PK1 cells expressing A2B-ARs were grown on permeable filter supports, and apical and basolateral membrane fractions were isolated by biotinylation. As show in Fig. 4, no detectable immunoreactivity was observed in the apical fraction of untransfected (−) LLC-PK1 cells; however, a faint band was apparent in the apical
fraction of transfected (+) LLC-PK1 cells. In contrast, when LLC-PK1 cells were transfected with α2B-ARs, a dense band at ~45 kDa was detected in the basolateral membrane fraction of transfected cells but not in untransfected cells. The relative mobility of the protein identified by Western blot analysis is consistent with the mobility of α2B-ARs demonstrated in previous studies (10, 27, 30). Scanning densitometry of the ~45-kDa protein showed that ~87% of the surface α2B-ARs were expressed at the basolateral domain, whereas ~13% of the receptors were expressed at the apical domain. These studies support the conclusion that the lateral labeling apparent by immunocytochemistry in Fig. 3 is consistent with a basolateral localization of α2B-ARs.

Steady-state expression of α2B-ARs at the basolateral membrane increases basolateral adenylyl cyclase activity. To determine whether the increase in expression of α2B-AR at the basolateral domain of LLC-PK1 cells regulated its effector, adenylyl cyclase, we stimulated apical and basolateral membrane adenylyl cyclase separately with forskolin. In separate wells, apical surfaces and basolateral surfaces of untransfected LLC-PK1 cells or LLC-PK1 cells expressing basolateral α2B-ARs were incubated with forskolin. cAMP accumulation was measured as an index of adenylyl cyclase activity. Basal levels of cAMP accumulation were 11.1 ± 2.83 and 6.6 ± 0.56 pmol/mg protein for untransfected LLC-PK1 cells and LLC-PK1 cells transfected with α2B-ARs, respectively (n = 3; P = NS). Stimulated levels of cAMP accumulation following the application of forskolin separately to apical and basolateral sides of untransfected cells were 113.8 ± 14.63 (n = 5) and 67.6 ± 13.6 (n = 6) pmol/mg protein (P < 0.05), respectively. Unlike untransfected cells, basolateral addition of forskolin in LLC-PK1 cells expressing basolateral α2B-ARs produced a dramatic increase in cAMP accumulation. When forskolin was applied to the apical surface of α2B-AR transfected cells, cAMP accumulation was similar to that observed in untransfected cells. In α2B-AR transfected LLC-PK1 cells, cAMP accumulation in response to separate apical and basolateral incubation with forskolin was 134.2 ± 8.41 (n = 5) and 471.6 ± 97.89 (n = 9) pmol/mg protein (P < 0.05), respectively.
Table 1 summarizes the effects of forskolin on cAMP accumulation in LLC-PK1 cells transfected with α2B-ARs. These observations suggested that the steady-state expression of α2B-ARs at the basolateral domain of LLC-PK1 cells influenced the activity of adenylyl cyclase at or near the basolateral domain.

To establish that α2B-ARs expressed at the basolateral membrane were coupled to adenylyl cyclase, we performed parallel experiments in which we incubated the basolateral membranes with forskolin and UK-14304, an α2-selective agonist. In comparison to the basolateral addition of forskolin alone, UK-14304 and forskolin reduced cAMP accumulation to 130.7 ± 25.74 pmol/mg protein (n = 9; P < 0.005), a level comparable to that observed in untransfected cells. This inhibitory effect of UK-14304 on forskolin-stimulated cAMP accumulation represented a fractional reduction of 0.71 ± 0.05. Table 1 summarizes the effects of UK-14304 on forskolin-stimulated cAMP accumulation represented a fractional reduction of 0.71 ± 0.05. Table 1 summarizes the effects of forskolin on cAMP accumulation in LLC-PK1 cells transfected with α2B-ARs. These observations suggested that the steady-state expression of α2B-ARs at the basolateral domain of LLC-PK1 cells influenced the activity of adenylyl cyclase at or near the basolateral domain.

To establish that α2B-ARs expressed at the basolateral membrane were coupled to adenylyl cyclase, we performed parallel experiments in which we incubated the basolateral membranes with forskolin and UK-14304, an α2-selective agonist. In comparison to the basolateral addition of forskolin alone, UK-14304 and forskolin reduced cAMP accumulation to 130.7 ± 25.74 pmol/mg protein (n = 9; P < 0.005), a level comparable to that observed in untransfected cells. This inhibitory effect of UK-14304 on forskolin-stimulated cAMP accumulation represented a fractional reduction of 0.71 ± 0.05. Table 1 summarizes the effects of UK-14304 on forskolin-stimulated cAMP accumulation represented a fractional reduction of 0.71 ± 0.05. Table 1 summarizes the effects of forskolin on cAMP accumulation in LLC-PK1 cells transfected with α2B-ARs. These observations suggested that the steady-state expression of α2B-ARs at the basolateral domain of LLC-PK1 cells influenced the activity of adenylyl cyclase at or near the basolateral domain.

To establish that α2B-ARs expressed at the basolateral membrane were coupled to adenylyl cyclase, we performed parallel experiments in which we incubated the basolateral membranes with forskolin and UK-14304, an α2-selective agonist. In comparison to the basolateral addition of forskolin alone, UK-14304 and forskolin reduced cAMP accumulation to 130.7 ± 25.74 pmol/mg protein (n = 9; P < 0.005), a level comparable to that observed in untransfected cells. This inhibitory effect of UK-14304 on forskolin-stimulated cAMP accumulation represented a fractional reduction of 0.71 ± 0.05. Table 1 summarizes the effects of UK-14304 on forskolin-stimulated cAMP accumulation represented a fractional reduction of 0.71 ± 0.05. Table 1 summarizes the effects of forskolin on cAMP accumulation in LLC-PK1 cells transfected with α2B-ARs. These observations suggested that the steady-state expression of α2B-ARs at the basolateral domain of LLC-PK1 cells influenced the activity of adenylyl cyclase at or near the basolateral domain.

To confirm that the pattern of labeling observed by immunocytochemistry of A1-AdRs in transfected LLC-PK1 cells represented that of apical surface receptors, apical and basolateral surface membrane proteins isolated by biotinylation were subjected to Western blot analysis. First, we were interested in determining the relative mobility of the A1-AdRs in LLC-PK1 cells. Figure 6A shows the results of Western blot analysis using an anti-FLAG monoclonal antibody. A band of ~38 kDa was detected from LLC-PK1 cells transfected with the A1-AdR (lane 2) and not from untransfected

![Fig. 5. Immunolocalization of LLC-PK1 cells transfected with human A1-AdRs. LLC-PK1 cells were seeded and grown to confluence on 8-well chamber slides. Cells were fixed in PLP and incubated with an anti-FLAG monoclonal antibody (27 µg/ml) as described in METHODS. Primary antibody was followed by a biotinylated goat anti-mouse secondary antibody and avidin-FITC. Diffusely punctate immunofluorescence was observed with sparing of the lateral membranes.](http://ajprenal.physiology.org/DownloadedFrom/F887REGULATIONOFAdenylLYLCYCLASE/102020334/10/14/2017)
cells (lane 1). This relative mobility of the A1-AdR is similar to the band observed in membranes from HEK-293 cells that overexpressed A1-AdRs (lane 4). Furthermore, as expected the molecular mass was reduced following deglycosylation (lane 3). These results demonstrate that the mobility of the protein identified by anti-FLAG monoclonal antibody approximates that of A1-AdRs.

When apical and basolateral surface proteins were isolated by biontination, the anti-FLAG monoclonal antibody detected a diffuse band of ~38 kDa from the apical fraction of LLC-PK1 cells transfected with A1-AdRs (lane 3) (Fig. 6B). No apparent band of similar molecular weight was observed from the apical (lane 1) or basolateral (lane 2) fractions of untransfected cells or from the basolateral domain of transfected cells (lane 4). Scanning densitometry of the ~38-kDa band from these surface biontination studies showed that ~86% of the cell surface receptors were expressed at the apical membrane and ~14% were expressed at the basolateral membrane (although not readily apparent by visual inspection). These results confirmed that the pattern of labeling observed by immunocytochemistry was consistent with receptors localized to the apical domain of transfected LLC-PK1 cells.

Steady-state expression of A1-AdRs at the apical domain increases apical adenylyl cyclase activity. The dramatic basolateral response of adenylyl cyclase in cells transfected with the α2B-ARs led us to examine whether the polarized response to forskolin could be reversed when another inhibitory G protein-coupled receptor, A1-AdR, was expressed at the opposite domain. LLC-PK1 cells demonstrating stable expression of apical membrane A1-AdRs were grown on permeable filter supports. In separate wells, apical and basolateral membranes were incubated with forskolin and IBMX. In contrast to the results with LLC-PK1 cells transfected with the α2B-ARs the expression of A1-AdR at the apical membrane led to a large increase in cAMP following apical membrane but not basolateral membrane incubation with forskolin. cAMP accumulation following apical vs. basolateral incubation with forskolin was 1,140.7 ± 144.25 (n = 9) vs. 98.2 ± 10.64 pmol/mg protein (n = 9) (P < 0.001), respectively. In contrast, the response of the apical vs. basolateral membranes to forskolin in control untransfected cells was 176.5 ± 29.41 (n = 9) vs. 111.1 ± 10.94 pmol/mg protein (n = 9) (P = NS), respectively. Table 2 summarizes the effects of forskolin on cAMP accumulation in LLC-PK1 cells transfected with A1-AdRs. In an analogous but in an opposite manner to α2B-ARs, the steady-state expression of apical A1-AdRs in LLC-PK1 cells influenced the activity of adenylyl cyclase following forskolin stimulation.

The functional coupling of apical A1-AdRs was assessed in parallel experiments in which the effect of CPA, an A1-AdR agonist, on forskolin-stimulated cAMP
accumulation was determined. In comparison with apical addition of forskolin alone, forskolin plus CPA (1 µM) reduced forskolin-stimulated cAMP to 170.3 ± 32.93 pmol/mg protein (n = 6; P < 0.001), which represents a fractional reduction of 0.82 ± 0.05. Similar to the experiments with α2B-ARs, this finding suggested that a portion of A1-AdRs was expressed at or near the apical domain and was coupled to adenylyl cyclase.

DISCUSSION

The plasma membrane of polarized renal epithelial cells is characterized by domains that differ functionally and biochemically (for a review, see Refs. 5, 20). In the kidney, apical plasma membrane is exposed to plasma ultrafiltrate and thus has a large surface area composed of microvilli that contain specialized proteins that function mainly to reabsorb various ions, nutrients and water. For example, the Na/H exchanger in the apical membrane of proximal tubule cells is the major mechanism for sodium entry across this membrane. The plasma membrane on the basolateral cell surface is exposed to blood and contains proteins for basic cellular processes such as Na-K-adenosinetriphosphatase (Na-K-ATPase), transferrin receptors, and low-density lipoprotein receptors. The asymmetric distribution of these proteins suggests that unique functions are subserved by both apical and basolateral domains. The importance of maintenance of these distinct domains is emphasized by pathological conditions that result in the loss of polarity. For example, the abnormal targeting of Na-K-ATPase in polycystic kidney disease could contribute to the abnormal tubular fluid accumulation and cyst formation (28).

Accumulating evidence indicates that receptors are asymmetrically expressed in renal epithelial cells and suggests that compartmentalization of receptors to apical and basolateral domains could provide an important mechanism for segregating receptor function to their respective domains. Filtered catecholamines, adenosine, or angiotensin II could regulate ion transport by stimulating apical membrane receptors. Functional studies indicate that apical membranes of renal epithelial cells are responsive to catecholamines (8, 17) and adenosine agonists (6, 15), and other studies have demonstrated directly the expression of A1-AdRs (22) and α2B-ARs at the apical domain (16, 17). How apical receptors couple to adenylyl cyclase is not well known, since it is thought that this enzyme is expressed exclusively at the basolateral domain (24). One explanation to account for the coupling of apical G protein-coupled receptors to basolateral adenylyl cyclase is that apical receptors undergo endocytosis to couple to basolateral adenylyl cyclase (8). We thought that another possible explanation for the apparent lack of apical adenylyl cyclase in experiments in vivo could be the low abundance of receptors that couple to this enzyme at the apical membrane. We wondered whether receptor expression regulates the degree of enzyme activity to which receptors are coupled.

To determine whether receptors that are expressed at opposite domains have the capability to regulate the activity of their effectors, we employed two receptors that have been shown to be targeted to opposite domains in renal epithelial cells (22). α2B-ARs (10) have been shown to be expressed at basolateral domains of renal epithelial cells. In contrast, canine A1-AdRs, when expressed in MDCK and LLC-PK1 cells, are localized primarily to the apical domain (22). We used this information and transfected LLC-PK1 cells with cDNAs encoding the rat α2B-AR and human A1-AdR to determine whether adenylyl cyclase activity is regulated by G protein-coupled receptors expressed at opposite domains.

Morphological and biochemical studies performed with LLC-PK1 cells transfected with the α2B-AR and A1-AdR confirmed the anticipated localization of these two receptors in our cell culture system. Immunofluorescence demonstrated α2B-AR immunoreactivity at lateral borders, consistent with findings of the localization of the α2B-ARs in rat kidneys (10) and in transfected renal epithelial cells (29). Furthermore, our surface biotinylation studies demonstrated that the majority of surface α2B-ARs were expressed at the basolateral domain.

In a similar manner, both our immunofluorescence and biotinylation studies demonstrated that the A1-AdRs were expressed primarily at the apical plasma membrane of LLC-PK1 cells. It is interesting to note that these results are similar to those of Saunders et al. (22), despite the fact that we used the human ortholog of A1-AdR. Moreover, functional evidence in A6 cells indicates apical localization of amphibian A1-AdRs (6).

Our functional studies further substantiated our biochemical and morphological studies. UK-14304, an α2B-Selective agonist, when applied to the basolateral membranes, produced a decrease in forskolin-stimulated cAMP accumulation. Thus, taken together, our immunohistochemistry and Western blot analysis not surprisingly provide evidence for the presence of α2B-ARs at the cell surface, thus confirming findings described previously (29). The results presented in the
current study showed that α2B-ARs expressed at or near the basolateral membrane surface were coupled to adenylyl cyclase through inhibitory G proteins. In a similar manner but at the opposite domain, we showed that apical A1-AdRs were coupled to adenylyl cyclase at or near the apical membrane surface.

The results of our experiments assessing adenylyl cyclase activity at apical and basolateral domains are noteworthy for two reasons. First, our results provide indirect evidence for the local interaction of receptors and the effector, adenylyl cyclase. The expression of apical A1-AdRs led to a dramatic increase in apical adenylyl cyclase activity in response to forskolin. Furthermore, the rise in cAMP accumulation in response to apically applied forskolin was inhibited to a large degree by the coapplication of an A1-agonist, CPA. Similar but opposite results were obtained with LLC-PK1 cells transfected with α2B-ARs. We believe that our results are consistent with the possibility that both A1-AdRs and adenylyl cyclase are coexpressed at the apical domain and α2B-ARs and adenylyl cyclase are coexpressed at the basolateral domain. The spatially restricted arrangement of receptor and effector could result in local receptor/effector coupling and obviate the need to invoke more complex mechanisms (8).

Although forskolin is a lipid-soluble molecule and could interact with adenylyl cyclase expressed at distant sites, we believe that the effects were limited to adenylyl cyclase at or near the domain to which it was applied. In other cells, forskolin has been demonstrated to act locally on adenylyl cyclase and not on adenylyl cyclase expressed in other regions. Jurevicius and Fischmeister (11) recently examined the effects of 30 µM forskolin on adenylyl cyclase-induced cAMP accumulation. They found that the effects of forskolin were limited to the region to which forskolin was applied. Diffusion of forskolin to distant sites was not observed. Furthermore, in our experiments, if diffusion of forskolin had occurred, we would have seen similar effects on adenylyl cyclase whether applied to apical or basolateral domains. Instead, we observed a polarized response that was most pronounced when forskolin was applied to the side to which recombinant receptors were expressed. To determine whether the effect of forskolin on cAMP accumulation was due to a distant effect of forskolin on adenylyl cyclase expressed at the opposite domain, we directly incubated the opposite domain with forskolin. As shown in Tables 1 and 2, the effect of forskolin when applied to plasma membrane domains that did not express recombinant receptors was similar to the corresponding domain in untransfected cells. Figure 7 illustrates the asymmetric response of forskolin in LLC-PK1 cells transfected with A1-AdRs or α2B-ARs. The apical/basolateral ratio of cAMP accumulation following application of forskolin was 1.57 ± 0.26 for untransfected LLC-PK1 cells. When recombinant A1-AdRs were expressed in the apical membrane, the apical-to-basolateral ratio of cAMP was 11.61 ± 1.47, an approximately sixfold increase in apical/basolateral ratio of cAMP accumulation compared with untransfected cells. In a similar manner but opposite in orientation, the basolateral/apical ratio of cAMP accumulation was greater in transfected than untransfected LLC-PK1 cells. After application of forskolin to untransfected LLC-PK1 cells, the basolateral/apical ratio was 0.4 ± 0.13. Transfection of recombinant α2B-ARs led to an increase of basolateral/apical ratio to 3.52 ± 0.73, an approximately eightfold increase above the ratio in untransfected LLC-PK1 cells. These results suggested that the asymmetric response to forskolin is consistent with an effect at or near the domain to which receptors were expressed.

For these reasons we believe that our results suggest that adenylyl cyclase is expressed and regulated at or near the apical or basolateral membranes. Definite proof will require separation of apical and basolateral membranes with intact adenylyl cyclase to permit detection of activity or immunoreactivity. The low abundance of adenylyl cyclase compared with other components of the adenylyl cyclase signaling cascade (1) makes these experiments very difficult. Indeed, this has been our experience in our attempts to demonstrate directly adenylyl cyclase activity or immunoreactivity from isolated apical and basolateral membranes.

Our results raise interesting issues that will need to be addressed in additional studies. First, it is interesting to speculate on the mechanism that might be responsible for this observation. It is possible that there is coordination of receptor and effector expression. Furthermore, because G proteins are heterogeneously localized on renal epithelia (25), polarized expression of receptors might influence the intracellular distribution of G proteins, leading to altered activity of adenylyl cyclase. Such coordinated expression of effects at specific cellular domains could lead to efficient cellular processing of external information. Alternatively, expression of inhibitory G protein-coupled receptors could
lead to sensitization of adenylyl cyclase (26). Sensitization refers to a process whereby exposure of tissues or cells to agents that inhibit adenylyl cyclase results in an increase in activity of adenylyl cyclase following removal of the agent. In our system, we used dialyzed FBS and therefore the presence of endogenous agonist is unlikely; however, inhibitory G protein-coupled receptors can be activated even in the absence of agonist. Previous studies have demonstrated that certain inhibitory G protein-coupled receptors are constitutively active (13) and could render adenylyl cyclase sensitized to the effects of forskolin.

Second, our results raise the possibility that multiple adenylyl cyclase isoforms could be expressed and compartmentalized in renal epithelial cells and participate in specific signaling events spatially restricted to separate domains.

We gratefully acknowledge Drs. Joel Linden (Department of Medicine, University of Virginia) and Diane L. Rosin and Kevin R. Lynch (Department of Pharmacology, University of Virginia) for providing valuable reagents and advice throughout the study. This work was supported in part from funds provided by the American Heart Association National Grant-in-Aid and Virginia Affiliate of the American Heart Association Grant-in-Aid. M. D. Okusa was a recipient of a National Kidney Foundation Clinical Scientist Award. Portions of this work have been previously published in abstract form (J. Am. Soc. Nephrol., vol. 7, p. 1312, 1996).

Address for reprint requests: M. D. Okusa, Division of Nephrology, Box 133, Univ. of Virginia Health Sciences Center, Charlottesville, VA 22908.

Received 29 May 1997; accepted in final form 31 July 1997.

REFERENCES