α₁- and β₂-Adrenergic Receptor Expression in the Madin-Darby Canine Kidney Epithelial Cell Line

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ABSTRACT The Madin-Darby canine kidney (MDCK) cell line, derived from distal tubule/collection duct, expresses differentiated properties of renal tubule epithelium in culture. We studied the expression of adrenergic receptors in MDCK to examine the role of catecholamines in the regulation of renal function. Radioligand-binding studies demonstrated, on the basis of receptor affinities of subtype-selective adrenergic agonists and antagonists, that MDCK cells have both α₁- and β₂-adrenergic receptors. To determine whether these receptor types were expressed by the same cell, we developed a number of clonal MDCK cell lines. The clonal lines had stable but unique morphologies reflecting heterogeneity in the parent cell line. Some clones expressed only β₂-adrenergic receptors and were nonmotile, whereas others expressed both α₁- and β₂-receptors and demonstrated motility on the culture substrate at low cell densities. In one clone, α- and β-receptor expression was stable for more than 50 passages. Catecholamine agonists increased phosphatidylinositol turnover by activating α₁-adrenergic receptors and cellular cyclic adenosine monophosphate accumulation by activating β₂-adrenergic receptors. Guanine nucleotide decreased the affinity of isoproterenol for the β₂-receptor but did not alter the affinity of epinephrine for the α₁-receptor. These results show that α₁- and β₂-receptors can be expressed by a single renal tubular cell and that the two receptors behave as distinct entities in terms of cellular response and receptor regulation. Heterogeneity of adrenergic receptor expression in MDCK clones may reflect properties of different types of renal tubule cells.

Catecholamines regulate a variety of cellular functions in the mammalian kidney, including tubule water and ion reabsorption, renin release, renal hemodynamics, and gluconeogenesis (20, 35). The influence of renal sympathetic nerves (which release catecholamines as neurotransmitters) on renal function has been discussed in several reviews (1, 6, 32). Catecholamines act on target cells by initially binding to cell surface receptors, and we and others (20, 41-43) have identified α₁-, α₂-, β₁-, and β₂-adrenergic receptors in membrane preparations of the kidney cortex by radioligand-binding techniques. However, the sites of action of adrenergic agents along the nephron have not been clearly defined. Determination of the location of catecholamine action within the kidney is particularly important to distinguish indirect adrenergic effects (resulting from changes in vascular tone) from direct effects on epithelial transport and metabolism. The kidney consists of segments that are anatomically and functionally distinguishable, yet each segment may contain several morphologically distinct cell types (8). To understand the regulation of renal function by catecholamines and sympathetic nerves, it therefore is important to study homogeneous populations of renal cells. Such populations may be found in the established renal cell lines, which have provided valuable information regarding kidney transport and metabolism (15).

We chose the Madin-Darby canine kidney (MDCK) cell line as a model system in which to examine the actions of catecholamines on renal tubule epithelium. The MDCK cell line was established in 1958 from normal dog kidney (12). MDCK cells retain differentiated properties of renal tubule...
epithelium, including transepithelial water and solute transport (28). These cells appear to be derived from distal tubule/collecting duct because of their enzyme markers (38), morphologic features (47), and antigenic determinants (16). Cells of the distal nephron are likely target sites for the catecholamines. The basolateral surface of the distal tubule is directly innervated by renal sympathetic fibers, and the distal nephron is the most responsive segment to β-adrenergic agonists as measured by cAMP accumulation (7). The presence of both α- and β-adrenergic responses in MDCK cells has been reported. In these cells, α-adrenergic agonists stimulate arachidonic acid metabolism (23) and K+ efflux (3). β-Adrenergic agonists increase cyclic adenosine monophosphate (cAMP) levels (38, 39) and transepithelial Cl- secretion (2). The adrenergic receptor subtype specificities of these responses have not been identified, although knowledge of the nature of these subtypes is important for understanding the mechanisms mediating target cell responses (11) and for identifying endogenous agonists and exogenous agonists and antagonists that will preferentially occupy the target cell receptors (1, 6, 20, 32, 35). Moreover, several investigators (22, 24, 37, 47) have presented evidence that MDCK is not a homogeneous cell line. We, therefore, addressed the following questions: (a) Are α- and β-adrenergic receptors present on renal tubule epithelium? (b) If so, which adrenergic subtype(s) are present? (c) Are α- and β-adrenergic receptors coexpressed by the same epithelial cell? (d) Are responses to α- and β-adrenergic agonists independently mediated?

In this paper, we describe the characterization of α- and β-adrenergic receptors in MDCK cells in terms of their ligand specificities, guanine nucleotide regulation, and intracellular second-messenger systems. We have developed a number of clonal MDCK cell lines with different morphologic features and different adrenergic receptor expression. We conclude that: (a) MDCK cells express α1- and β2-adrenergic receptors; (b) MDCK is a heterogeneous cell line consisting of at least two stable cell types, one with both α1- and β2-receptors and the other with only β2-receptors; (c) in a clonal cell line derived from parent MDCK cells, α1- and β2-agonists mediate discrete intracellular responses that are independently regulated. Preliminary reports of some of these findings have been presented in abstract form (29, 30).

MATERIALS AND METHODS

Materials: The following compounds were received as gifts from the sources indicated: (-)-epinephrine, (+)-epinephrine, and (-)-norepinephrine (+)-bitartrate salts (Sterling Winthrop Research Institute, Rensselaer, NY); (-)-propranolol HCl, (+)-propranolol HCl (Ayerst Laboratories, New York); phenotlamine mesylate, clonidine (Geigy Pharmaceutical, Summit, NJ); cyanopindolol HCl (Sandoz Inc., Basel, Switzerland); prazosin (Pfizer Inc., Groton, CT); practolol (ICI Americas Inc., Wilmington, DE); [3H]-[4-(4-hydroxyphenyl)ethylaminomethyl]tetralone HCl (Becton Dickinson & Company, Rutherford, New Jersey); [125I]iodocyanopindolol (ICYP) and [125I]iodo-2-[β-(4-hydroxyphenyl)ethylaminomethyl]tetralone ([125I]HEAT); Cyanopindolol (CYP) was iodinated by minor modification of the method of Engel et al. (10). The reaction mixture contained 20 μg CYP.
with 10 ml of 37°C incubation buffer. Radioactivity retained on the filters was determined using a Searle gamma counter (Searle Analytic, Inc., Des Plaines, IL) at 63% efficiency (129), or with 4.5 ml of scintillation cocktail in a Beckman scintillation counter (Beckman Instruments, Inc., Fullerton, CA) at 30% efficiency (11). Nonspecific binding was defined with the following agents: for [3H]prazosin and [3H]lyscrin, 10 μM phenotamine; for [125I]IHEAT, 0.5 or 1.0 μM prazosin; and for [125I]JICYP, 1.0 μM (S)-propranolol. Specific binding was determined by subtraction of nonspecific binding from total binding. The specific binding was routinely the following percent of the total binding at concentrations of the radioligands near their dissociation constants: >60% for [3H]prazosin, >70% for [125I]IHEAT and >75% for [125I]JICYP. Specific binding of the radioligands was linear with cell number. When catecholamines were used in binding experiments, ascorbic acid was included at a final concentration of 0.5 mg/ml to prevent drug oxidation. Replacement data points varied by <10%.

Data Analysis: The dissociation constant (Kd) and maximum number of binding sites (Bmax) were determined from Scatchard analysis (10) of saturation binding isotherms. The line of the Scatchard plot was fitted by linear regression analysis.

Competitive binding curves were analyzed by a computer program (LI-GAND) that performs iterative nonlinear regression (33).

Phosphatidylinositol Assay: MDCK-D cells were seeded in 35-mm culture dishes containing 3 ml of medium and grown to confluence (2 d). Assays were performed with duplicate dishes in a humidified 37°C incubator with a 5% CO2 atmosphere. Experiments were initiated by rinsing each dish twice with 20 ml of 37°C Krebs-Henseleit buffer (KH: 118 mM NaCl, 4.7 mM KCl, 3.0 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 0.5 mM EDTA, 25 mM NaHCO3, and 10 mM glucose, pH 7.4) and then adding 750 μl of myo-[3H]inositol (6.6 Ci/mmol, ~4.5 μCi/dish) in KH. 60 min later, 7.5 μl of drug or control solution was added, and the incubation was continued for an additional 30 min. Ascorbic acid (5 mg/ml) was included in the freshly prepared epinephrine stock solutions to prevent drug oxidation before addition to the cells (final concentration, 0.05 mg/ml). Incubations were terminated by removal of the incubation mixture by aspiration followed by three washes with 2 ml of ice-cold saline. Cold saline (0.5 ml) was then added, and the cells were removed from each dish by scraping. Each dish was washed two more times with 0.5 ml of ice-cold saline, and the pooled washes were centrifuged for 30 s in a microfuge. The supernatant was removed, and the 0.75 ml of ice-cold chloroform/methanol (1:2) was added to the pellet. The termination procedure was completed in <3 min.

Phosphatidylinositol (PtdIns) was extracted by the addition of 0.2 ml of 2 M KCl to each sample (final proportion of chloroform/methanol/2 M KCl, 10:5:4) followed by sonication using a Kontes cell disrupter in three 10-s bursts. A two-phase system was formed by the addition of 0.25 ml of chloroform and 0.25 ml of 2 M KCl followed by centrifugation at 1,600 g for 20 min at 4°C. The aqueous upper phase and any interfacial material were removed, the organic lower phase was decanted, and the remaining tissue pellet was discarded. The lower phase was washed twice with 0.5 ml of chloroform/methanol/water (3:4:7) and dried under N2, and the tritium content was determined in 3.5 ml of OCS scintillation cocktail (Amersham/Triton X-100 (2:1). Control experiments showed that <0.05% of the free myo-[3H]inositol contaminated the lower phase and that 100 ± 1% of the total lower phase counts were recovered as PtdIns, as determined by lipid separation by two-dimensional thin-layer chromatography (first dimension: chloroform/methanol/water/18 M NH4OH, 130:70:8:0.5; second dimension: chloroform/acetone/methanol/acetic acid/water, 10:4:2:2:1) on 20 x 20 cm silica gel plates (Merck & Co., Rahway, NJ) (36).

CAMP Assay: MDCK cells were grown to confluence (1-3 d; 3-5 x 105 cells/well) in 24-well culture dishes. The medium was removed by aspiration, and the cells were washed twice with 1 ml of Hank's balanced salt solution. Incubation medium (0.4 ml) consisting of a freshly prepared solution of drugs in HBSS was then added, and the dishes were incubated at 37°C for 5 min. At the conclusion of the incubation, 100 μl of 40% trichloroacetic acid was added to each well. After a 10-min incubation at room temperature, the trichloroacetic acid extracts were removed from each well and transferred to 0.5 ml Dowex AG 1 x 8 columns. The columns were washed with 2.5 ml of water, and the CAMP was eluted with 4 ml water. Recovery of the CAMP was 90-95% as determined in control columns using [3H]CAMP. The cAMP eluate was dried with a Speedvac concentrator (Savant Instruments, Inc., Hicksville, NY). Cyclic AMP was measured by a competitive binding protein assay using aliquots of the dried sample fractions resuspended in sodium acetate buffer (18, 19).

RESULTS

MDCK Cell Lines

The results of our initial radioligand binding experiments with MDCK membranes indicated that MDCK cells possessed both α1- and β2-adrenergic receptors. These receptors are described in subsequent sections. We found, however, that the expression of α1-receptors varied in MDCK cell lines obtained from different sources. Our first experiments were conducted using MDCK cells derived from ATCC stock culture that had been maintained in the laboratory of Dr. Saier. These cells had approximately equal numbers of α1- and β2-receptors (Table I). When we obtained cells directly from ATCC, we found that early passage cultures had more

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**TABLE 1**

| Cell Line | Origin | Morphology | Domes | Alpha1 | Beta2 |
|-----------|--------|------------|-------|--------|-------|
| Parental: | ATCC   | ATCC, p53  | M and NM | +      |       |
|           | ATCC   | M. Saier   | M, F  | +      |       |
| Clonal:   | C      | ATCC, p59  | NM, F | +      |       |
|           | D      | ATCC, p59  | M, F  | ±      |       |
|           | H      | ATCC, p68  | NM, C | ±      |       |
|           | L      | ATCC, p68  | NM, C | ±      |       |
|           | N      | ATCC, p68  | NM, F | -      |       |
|           | Q      | M. Saier   | M, F  | +      |       |
|           | R      | ATCC, p59  | M, F  | +      |       |
|           | S      | ATCC, p68  | M, F  | +      |       |
|           | T      | ATCC, p68  | M, F  | +      |       |
|           | U      | ATCC, p68  | M, F  | +      |       |

The origin column indicates the cell line from which each cell line was derived. The morphology column refers to the appearance of the cells when seeded at low density. "Domes" refers to the presence or absence of multicellular domes (or hemicylinders) in confluent monolayers as observed over ~20 passages of continuous growth. The number of sites for both adrenergic receptor subtypes was determined in parallel at the indicated passage number by Scatchard analysis of radioligand-binding isotherms as described in the text. ATCC, American Type Culture Collection; SF, ATCC stock from Saier's laboratory grown in serum-free medium for 6 mo and then returned to serum-containing medium; NM, nonmotile; M, motile; F, flattened; C, cuboidal; p, passage number, for clonal cell lines this refers to the number of passages after cloning.

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\[ \text{\textbullet~2\text{-receptors than \textgreek{o}\text{-receptors (Table I) and that the number of \textgreek{o}\text{-receptors decreased with increasing passage number. The two cell lines were maintained in our laboratory under identical culture conditions. To resolve the problem of variable \textgreek{o}\text{-receptor expression, we cloned early-passage ATCC cells (MDCK-ATCC) by limiting dilution and obtained several clonal cell lines. In addition, we cloned cells that we had originally obtained from Dr. Saier's laboratory but that had been grown in serum-free medium for ~6 mo and then returned to serum-containing medium (MDCK-SF).}

Phase-contrast micrographs of several representative clonal lines are shown in Fig. 1. When ATCC cells were seeded at low density, cells with different morphologies were observed within the population (Fig. 1A). Some colonies consisted of cells that were elongated and flattened, while others contained more cuboid cell types. Clones derived from parent populations were homogeneous but exhibited distinct morphologic features resembling different elements of the parent population. The morphologic differences between clones were most pronounced in subconfluent cultures and were not as obvious in cells seeded at near-confluent densities or in confluent cultures. Clone R and other clones derived from MDCK-SF had a typical epithelioid appearance (Fig. 1B and C). At low density, these clones were extended, flattened, and motile. Upon reaching confluency, the cultures formed multicellular "domes," which is indicative of apical to basolateral fluid transport (22) (Fig. 1C). These clones were extremely resistant to detachment from their culture substrate by trypsin-EDTA at confluency. A clone with this same general morphology was also obtained from early passage MDCK-ATCC (clone D; Fig. 1D). However, clone D seldom formed domes under routine culture conditions. Other clonal lines obtained from MDCK-ATCC had strikingly different morphologies. These cells were nonmotile, as evidenced by their growth in discrete, compact colonies at low cell densities. Some, such as clone L (Fig. 1F), were cuboid and did not spread on the culture substrate. Others, such as clone M (Fig. 1E), were more flattened but were similarly nonmotile. The majority of the nonmotile clones did not form structures that could be identified as domes by phase-contrast light microscopy. In addition, these clones were relatively easily detached from their substrate with trypsin-EDTA, even after attaining confluency. When cultures were initiated from clonal lines that had been stored at ~70°C for several months, the morphologies of the thawed cells were identical to those of the original clone, indicating that the clonal morphologies were stable phenotypic features.

Clonal MDCK cell lines were screened for their expression of \textgreek{o}\text{- and \textgreek{2}-adrenergic receptors by radioligand binding techniques (described in the following sections). Scatchard analyses of radioligand binding isotherms were performed at least once with each clone for both \textgreek{o}\text- and \textgreek{2}-receptors. As summarized in Table I, we found that the nonmotile clones did not express \textgreek{o}\text-receptors, as shown by their lack of detectable specific binding of the iodinated \textgreek{o}\text-receptor probe, \textgreek{25I}IHEAT. The lower limit of detection was ~100 \textgreek{o}\text-receptors per cell. The motile cell lines consistently expressed \textgreek{o}\text-receptors, and all cell lines expressed \textgreek{2}-receptors. There were 8,000–20,000 \textgreek{o}\text- and 500–6,000 \textgreek{2}-receptors per cell in receptor-bearing cells. Growth of Saier's ATCC cells in serum-free defined medium for 2 mo had no acute effect on receptor expression, since \textgreek{o}\text- and \textgreek{2}-receptor numbers on serum-free cells (4,600 \textgreek{o}\text- and 4,000 \textgreek{2}-receptors/cell) were comparable to those of cells grown in serum-containing medium (5,000 \textgreek{o}\text- and 3,900 \textgreek{2}-receptors/cell) when assayed in parallel. Therefore, we attribute the high frequency of \textgreek{o}\text-receptor-
bearing clones in MDCK-SF primarily to the composition of the parent cell population. However, after long-term (5 mo) growth in serum-free medium, the MDCK-SF cells had 13,000 α1-receptors and 2,400 β2-receptors per cell. Therefore, we cannot rule out the possibility that the increased frequency of α1-receptor-bearing cells resulted, in part, from selective pressure caused by long-term growth in serum-free medium.

To study both α1- and β2-adrenergic receptors in a homogeneous cell population, we used clone D. This clone was chosen because it was derived from the early-passage ATCC stock and was therefore most likely to reflect properties of the renal cell from which it was derived. The morphology and adrenergic receptor expression of clone D have been stable for over 50 passages since initial cloning. In the following sections we will describe adrenergic receptor properties in both parental (MDCK-ATCC) and clonal (MDCK-D) lines.

α1-Adrenergic Receptor Characterization

Two subtype-selective α-adrenergic antagonists, [3H]prazosin and [3H]yohimbine, which we have found useful in identifying renal α1- and α2-receptors, respectively (42), were used in initial binding experiments with parental MDCK cells. No specific binding of [3H]yohimbine was detected in several experiments, indicating that MDCK cells did not express α2-adrenergic receptors. In contrast, specific binding of [3H]prazosin was consistently observed, suggesting that α1-recep-

![Graph](https://via.placeholder.com/150)

**Figure 2** Kinetic analyses of [3H]prazosin and [125I]IHEAT binding to MDCK membranes. Specific binding of 0.50 nM [3H]prazosin (upper panel) to MDCK and 0.13 nM [125I]IHEAT (lower panel) to MDCK-D membranes at 37°C was followed as a function of time (filled circles). After 60 min, the rate of dissociation of the radioligands (circles) was followed subsequent to the addition of phentolamine to a final concentration of 10 μM (for [3H]prazosin, upper panel) or prazosin to a final concentration of 1 μM (for [125I]IHEAT, lower panel). Each data point represents the mean of duplicate samples. The data are expressed as percent of maximum specific binding as defined in the text.

![Graph](https://via.placeholder.com/150)

**Figure 3** Saturation binding isotherms for [3H]prazosin and [125I]IHEAT on MDCK membranes. MDCK membranes were incubated with the indicated concentrations of [3H]prazosin (upper panel) or [125I]IHEAT (lower panel) for 60 min at 37°C. Conditions for the binding assay and the determination of specific binding are described in the text. The insets show the Scatchard analyses of the binding data used to determine the equilibrium dissociation constants (Kd), binding site numbers (Bmax), and Hill coefficients. These data were obtained in a single experiment using the same membrane preparation for both radioligands. Each data point represents the mean of triplicate determinations.

Further characterization of the specific [3H]prazosin binding sites showed that these sites demonstrated properties expected for α1-adrenergic receptors. A kinetic analysis of [3H]prazosin binding to MDCK membranes is shown in Fig. 2 (upper panel). Specific binding reached equilibrium rapidly at 37°C, with saturation occurring in 20 min. Upon addition of 10 μM phentolamine (the α1-adrenergic antagonist used to define nonspecific binding), specific [3H]prazosin binding dissociated with a half-time of 18 min and was totally reversible. The specific binding of [3H]prazosin was saturable, as shown in Fig. 3 (upper panel). In this representative experiment, the maximum number of binding sites (Bmax) was 5,700 sites per cell. Scatchard analysis of the equilibrium binding isotherm indicated the presence of a single class of binding sites with a Kd of 0.74 ± 0.3 nM (n = 4). The noncooperative nature of the binding sites was indicated by a Hill coefficient of unity (1.08 ± 0.09). When [3H]prazosin binding assays were performed in the presence of competing adrenergic ligands, the results were consistent with binding to a pure population of α1-receptors (Fig. 4). Indoramin, a classic α1-antagonist, had
an affinity for the [3H]prazosin binding sites 19 times higher than did the α2-antagonist yohimbine. Using the LIGAND curve-fitting program, we were able to determine whether competing drugs bound to a single site with one affinity (“one-site fit”) or whether the binding was better described by two (or more) sites with different affinities (“two-site fit”) (33). The subtype-selective antagonists bound to a single population of receptors, inasmuch as fits of the competition binding data for these drugs based on a two-site model were not significantly improved over fits to a one-site model. [3H]Prazosin bound to stereoselective sites, as evidenced by the severalfold higher affinity of (-)-epinephrine as compared with (+)-epinephrine; this result is as expected for α-adrenergic receptors (42).

β2-Adrenergic Receptor Characterization

β-Adrenergic receptors were identified on MDCK membranes with [125I]ICYP, a high-affinity β-antagonist that binds with equal affinity to both β1- and β2-receptor subtypes (10). The kinetics of association and dissociation of [125I]ICYP from MDCK membranes is shown in Fig. 5. [125I]ICYP specific binding reached equilibrium in 40 min at 37°C and was slowly dissociable (t1/2 ~ 300 min) upon the addition of 1 μM propranolol. [125I]ICYP specific binding was saturable, with maximum binding to 2,100 sites/cell in this representative experiment. Scatchard analysis of the equilibrium binding isotherm (Fig. 6) showed a single class of noncooperative binding sites with a Kd of 0.051 ± 0.03 nM (n = 12) and a Hill coefficient of 1.00 ± 0.09. The results of competitive binding assays are shown in Fig. 7 and are summarized in Table II. The [125I]ICYP binding sites were determined to be β2-receptors on the basis of the dissociation constants of catecholamine agonists (isoproterenol < epinephrine < norepinephrine). The subtype-selective antagonists practolol (β1) and IPS 339 (β2) each bound to a single class of binding sites, since their competition binding data were adequately described by one-site models. From these results, we conclude that MDCK cells have a pure population of β2-receptors and have no detectable β1-receptors. (+)-Propranolol was 210 times more potent than (-)-propranolol, and (-)-isoproterenol was 23 times more potent than (+)-isoproterenol, indicating that the [125I]ICYP-binding sites had the stereoselectivity expected of β-adrenergic receptors.

Stability of Receptor Expression in Clone D

The presence of both α1- and β2-receptors on a number of clonal MDCK lines showed that these two receptor types were expressed on the same cell. The pharmacologic properties of the α1- and β2-receptors of clone D were similar to those of the parent cell lines (Table II). A subclone of clone D (D-1) also coexpressed α1- and β2-receptors, confirming that these receptors were present on a single cell. To use clone D as a model system in which to examine these receptors, we required evidence that the receptor expression was stable with time in culture. Scatchard analyses of radioligand binding to α1- and β2-receptors in early- and late-passage MDCK-D cells are shown in Fig. 8. Receptor numbers were compared between cells that had been maintained for 53 passages since initial cloning from the parent line and cells that had been grown from frozen early-passage stock and had been main-
### Table II
Summary of Equilibrium Dissociation Constants for Adrenergic Ligands in MDCK

| Radioligand | Competing Ligand | ATCC | Clone D |
|-------------|------------------|------|---------|
| **I. Alpha** |                  |      |         |
| A. \[^{3}H\]prazosin |                 |      |         |
| Antagonist: | Indoramin | 20   | —       |
|            | phentolamine | 140  | —       |
|            | yohimbine | 380  | —       |
|            | clonidine | 1,400 | —      |
|            | (-)-epinephrine | 6,200 | — |
|            | (-)-norepinephrine | 18,000 | — |
|            | (+)-epinephrine | 120,000 | — |
| Agonist:   | clonidine | 1,400 | —       |
|            | (-)-epinephrine | 6,200 | —     |
|            | (-)-norepinephrine | 18,000 | — |
|            | (+)-epinephrine | 120,000 | — |
| B. \[^{125}I\]IHEAT |                 |      |         |
| Antagonist: | prazosin | 0.048 | 0.076   |
|            | HEAT | — | 2.8     |
|            | phenoxybenzamine | — | 46 |
|            | phentolamine | 640 | — |
|            | yohimbine | 1,100 | 4,100 |
| Agonist:   | (-)-epinephrine | 2,100 | 2,200 |
|            | (+)-epinephrine | 87,000 | — |

**II. Beta-adrenergic receptors**

A. \[^{125}I\]ICYP

| Antagonist: | IPS 339 | 0.19   | —       |
|            | (-)-propranolol | 0.36   | —       |
|            | (+)-propranolol | 77     | —       |
|            | practolol | 7,200  | —       |
| Agonist:   | (-)-isoproterenol | 35     | 23      |
|            | (-)-epinephrine | 44     | 280     |
|            | (+)-isoproterenol | 800    | —       |
|            | (-)-norepinephrine | 3,300  | 3,000   |

$K_D$ for the radioligands were determined by Scatchard analysis of equilibrium binding isotherms; $K_D$s for unlabeled agonists and antagonists were determined from the concentrations that reduced specific radioligand binding by 50% and in all cases were estimated by the LIGAND program using a one-site model to fit the data. Each value was derived from a single experiment in which several other ligands were concomitantly tested, so that their rank orders of potency might be easily compared.

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**Figure 5** Kinetic analysis of \[^{125}I\]ICYP binding to MDCK membranes. Specific binding of 0.07 nM \[^{125}I\]ICYP to MDCK membranes at 37°C was followed as a function of time (filled circles). After 60 min, the rate of dissociation of \[^{125}I\]ICYP (circles) was followed subsequent to the addition of (±)-propranolol to a final concentration of 1 μM. Each data point represents the mean of duplicate determinations. The data are expressed as percent of maximum specific binding as defined in the text.

**Figure 6** Saturation binding isotherm of \[^{125}I\]ICYP to MDCK membranes. MDCK-D membranes were incubated with the indicated concentrations of \[^{125}I\]ICYP for 60 min at 37°C, and specific binding was determined as described in the text. The inset shows a Scatchard analysis of the equilibrium binding data, which was used to calculate the $K_D$, $B_{max}$, and Hill coefficient. Each data point represents the mean of triplicate determinations.

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Guanine Nucleotide Modulation of Agonist Binding

Guanine nucleotides are known to lower the affinity of β-adrenergic agonists for their receptors (44) and have been

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This experiment was repeated several times with both MDCK-D and MDCK ATCC cells and with both [3H]prazosin and [125I]IHEAT, yet no consistent effect of Gpp(NH)p was observed. The data shown are fit to a one-site model, because

shown to have a similar but less pronounced effect at renal α1-adrenergic receptors (42). We investigated guanine nucleotide effects on agonist binding to MDCK-D membranes to see whether the receptors were differentially sensitive to the nucleotide.

The effects of the nonhydrolyzable guanine nucleotide guanyl-5′-imidodiphosphate (Gpp(NH)p) on agonist-binding to MDCK-D α1- and β2-receptors are shown in Fig. 9. In the lower panel of Fig. 9, the effect of Gpp(NH)p on the affinity of isoproterenol for MDCK-D β2-receptors is shown. The control curve shown is the two-site fit, which was statistically superior to the one-site fit (P = 0.003) for these data. In this representative experiment, the high-affinity site represented 76% of the total sites, with a KD for isoproterenol of 15.5 nM. The low-affinity site (24% of total sites) had a KD of 490 nM. In the presence of guanine nucleotide, the data were described adequately by a one-site fit (P > 0.1 for two-site vs. one-site fit), with a KD for isoproterenol of 470 nM. These data are similar to observations in other β-receptor systems (44). The effect of guanine nucleotides is thought to reflect heterogeneity of agonist binding sites, in which some of the agonist-receptor complexes interact with a GTP-binding regulatory protein that mediates the coupling between receptor and adenylate cyclase (44). In the absence of added guanine nucleotide, this portion of the agonist-bound receptor population is in a high-affinity state that converts to a low-affinity state upon the addition of guanine nucleotide.

As shown in the upper panel of Fig. 9, Gpp(NH)p did not alter the affinity of (-)-epinephrine for MDCK-D α1-receptors (KD = 11,000 nM for control and 8,700 nM with Gpp(NH)p).
fits to a two-site model were not significantly better (P > 0.1 for two-site vs. one-site fit). These results show that MDCK α- and β-receptors are not affected in the same way by guanine nucleotides.

Phosphatidylinositol Turnover

The effect of epinephrine on the incorporation of [3H]inositol into phosphatidylinositol (PtdIns) was examined to determine whether α1-receptors in MDCK cells are functionally coupled to this putative second-messenger system, as has been observed for α1-receptors in other tissues (11). MDCK-D cells were prelabeled with myo-[3H]inositol for 60 min, exposed to various concentrations of (-)-epinephrine for an additional 30 min, and the incorporation of the label into cellular PtdIns was determined (Fig. 10). In the presence of epinephrine the rate of incorporation of [3H]inositol increased to a maximum of 92% above the baseline levels seen in the absence of epinephrine (Fig. 10). A half-maximum effect (EC50) occurred with 1.8 μM epinephrine, a concentration similar to the Ks of epinephrine in the binding studies (Table 2). The response to epinephrine was totally blocked by the simultaneous addition of 1 μM prazosin (Fig. 10), whereas prazosin alone had no effect on inositol incorporation (data not shown). Epinephrine stimulation of myo-[3H]inositol incorporation into PtdIns was also observed when cells were prelabeled with myo-[3H]inositol for 30 min, rather than 60 min, before drug addition (data not shown). These results indicate that α1-receptor occupation stimulates PtdIns synthesis in MDCK cells.

Cyclic AMP Accumulation

The effect of isoproterenol on cyclic AMP accumulation in MDCK cells was studied to determine whether the β-receptors were coupled to adenylate cyclase. When ATCC-derived cells were incubated with isoproterenol, a rapid increase in cAMP accumulation was observed. In the presence of 0.5 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, the cAMP attained a maximum level after 5 min of isoproterenol exposure (data not shown). The dose-response relationship for this effect (Fig. 11, left) indicated that the EC50 for isoproterenol was 0.1 μM. Epinephrine also increased cAMP accumulation, and this increase was completely blocked by 1 μM propranolol (Fig. 11, right). Propranolol alone had no effect on cAMP levels. Isoproterenol increased cAMP levels to a similar extent in several clonal cell lines. The increase in cAMP content over baseline levels in response to 10^{-5} M isoproterenol was 978, 1,030, and 1,077 pmol cAMP/10^7 cells/10 min for clones D, L, and N, respectively. These results show that enhanced cAMP accumulation was promoted by agonist occupancy of β-adrenergic receptors in MDCK cells.

DISCUSSION

In this study we have shown that MDCK renal epithelial cells have α- and β-adrenergic receptor subtypes, that both receptor types can be expressed by a single cell, that the receptors show different sensitivity to guanine nucleotides, and that α1- and β-receptors mediate distinct intracellular responses. The radioligand binding experiments showed that α- and β-receptors of MDCK can be classified as classic α1- and β-receptors and that there are no detectable α2- or β2-receptors. In contrast, studies of membranes prepared from kidney cortex have indicated that the number of α2- and β1-receptors is two to three times greater than that of α1- and β2-receptors (41-43), suggesting that MDCK cells represent only a minor portion of the adrenergic receptor-bearing cells in the kidney cortex. Our findings suggest that growth of MDCK in vitro does not alter the recognition properties of these receptors, inasmuch as the receptors show binding specificities characteristic of classic α1- and β2-adrenergic receptor subtypes. In this regard, it is interesting that the affinity of the endogenous agonists norepinephrine and epinephrine for β2-receptors is much higher than that for α1-receptors of MDCK cells.

The findings further our understanding of the nature of adrenergic receptors in renal tubules. The functions of renal α1-receptors are poorly understood, and these receptors have been thought to be located primarily on the renal vasculature (20, 41). However, recent studies indicate that renal α1-receptors mediate gluconeogenesis (27), which probably occurs in
tubule cells (17, 26). Other work suggests that renal tubule $\alpha$-receptors may regulate ion and water reabsorption by the nephron (6, 20, 32, 35). The results of our experiments with MDCK cells provide direct evidence that $\alpha_1$-receptors are located on renal tubule epithelium. Further, although data indicating that $\beta$-adrenergic receptors are present on tubule epithelium have been presented (7, 20, 31), we show that tubule cells can possess a "pure" population of $\beta_2$-adrenergic receptors. Our findings imply that the administration of drugs acting at $\alpha_1$- and $\beta_2$- adrenergic receptors may perturb tubule cell function. Because $\alpha_1$- and $\beta_2$-receptor subtypes are present on several clonal isolates from parent MDCK cells, at least some (but probably not all) tubule cells are able to express both receptor subtypes. Additional work will be required to determine exactly which tubular cells in vivo possess one or both types of adrenergic receptors.

Previous work has indicated that $\alpha$- and $\beta$-adrenergic receptors are regulated quite differently by guanine nucleotides and other factors in target cells. However, few studies have been performed with cells of which one could be confident that both classes of receptors are simultaneously expressed (18, 34). As observed in other systems (44), Gpp(NH)p shifted the high-affinity component of agonist binding to $\beta$-adrenergic receptors of MDCK cell membranes to a lower affinity, such that only low-affinity sites were detected. In contrast, epinephrine bound to a single class of $\alpha_1$-receptor sites on MDCK membranes, and the affinity of these sites for agonist was not altered by guanine nucleotide. $\alpha_1$- Receptors are not generally thought to be coupled to adenylyl cyclase or to a guanine nucleotide regulatory protein. However, in other $\alpha_1$-receptor systems guanine nucleotides have been shown to decrease agonist affinity (14, 22). $\alpha_2$- Receptors of MDCK cells appear to be in a homogeneous (pseudo-Hill slope for epinephrine = 0.87 ± 0.05 [n = 3]), low-affinity state in the absence of guanine nucleotide, and this may account for the lack of guanine nucleotide effect. This lack of regulation of $\alpha_1$-receptors by guanine nucleotides may reflect properties of the native receptors or may be secondary to the membrane preparation or incubation conditions.

In addition to differences in receptor regulation by guanine nucleotides, our findings show important differences in the linkage of $\alpha_1$- and $\beta_2$-receptors to second-messenger systems. Activation of adenylyl cyclase by catecholamines in renal membranes and in MDCK cells has been correlated with changes in transepithelial transport (20, 35). We have confirmed that adenylyl cyclase activation is mediated by $\beta$- receptors (in MDCK, by $\beta_2$-receptors). $\alpha_1$-Adrenergic regulation, in a variety of systems, results in calcium mobilization and alterations in membrane phospholipid metabolism (11). The stimulation of PtdIns turnover by $\alpha_1$-agonists in MDCK may be a key step in the events resulting from $\alpha_1$-receptor occupation. $\alpha$-Adrenergic stimulation of PtdIns synthesis is thought to result from initial stimulation of PtdIns hydrolysis (11), which may result in the release of arachidonic acid, which may in turn result in increased prostaglandin synthesis. $\alpha$-Adrenergic-induced changes in prostaglandin synthesis have been reported for MDCK cells (23). Our data suggest that alterations in PtdIns metabolism may be an initial step in this response.

In spite of differences in receptor recognition properties in and linkage to second-messenger systems, it has been suggested that $\alpha$- and $\beta$-adrenergic receptors may be located at separate sites on the same protein or may be interconver-

tible entities (21). Most recently, receptor interconversion has been suggested as a mechanism to account for temperature-induced changes in adrenergic effects on renin release by the kidney (5). Thus far, we have found no evidence for receptor interconversion in clonal MDCK cells. MDCK cells can express both $\alpha_1$- and $\beta_2$-receptors or only $\beta_2$-receptors. The receptors behave as distinct macromolecules in terms of their modulation by guanine nucleotide, apparently because they are coupled to different regulatory proteins. Each receptor mediates discrete biochemical responses: $\alpha_1$-agonists increase PtdIns turnover, and $\beta_2$-agonists increase cAMP levels, indicating that the receptors likely are discrete entities. In additional studies we have obtained preliminary data on receptors solubilized from clone D, indicating structural differences in $\alpha_1$- and $\beta_2$-receptors from these cells (45).

In this study we also obtained information regarding the heterogeneity of the MDCK cell line showing that MDCK clones differ in morphology and in adrenergic receptor expression. Expression of $\alpha_1$-receptors was observed only in motile clones but was not correlated with the ability of clones to form domes. Clonal morphologies and receptor expression were stable phenotypic features, which suggests that the various cell types were present in the parent cell line and did not arise by spontaneous mutation. Morphologic differences between clonal MDCK lines have been observed by others (22, 47). Differences in ion transport (37) and arachidonic acid metabolism (24) between different nonclonal MDCK sublines have been noted. Variations between nonclonal MDCK sublines may have arisen by selection for one cell type over another during maintenance in culture. Inasmuch as MDCK was derived from a mince of kidney cortex (12), cell heterogeneity is not unexpected. MDCK cells appear to be derived mainly from distal tubule/collecting duct; however, heterogeneity of cell types exists even within these segments. Valentich (47) has suggested that two MDCK cell types may be derived from the principal and intercalated cells of the collecting duct. Our data confirm his conclusions regarding the stability of morphologically distinct clones, but additional studies will be required to draw further analogies between our clonal lines and the cell types identified by Valentich. In addition, we have not yet determined the cellular factor(s) that result in the expression of the motile phenotype. MDCK cells synthesize basal lamina when grown on collagen (47) and also release biomatrix components when grown on plastic (4). Clonal variations in the production of such molecules may affect cell attachment and motility, but this hypothesis will require further study. We believe that cloned MDCK cells will serve as unique model systems for further studies of the cellular regulation of renal adrenergic receptors and for evaluating other cellular properties of this cultured renal cell line.

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