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Renal α_1-Adrenergic Receptor Subtypes: MDCK-D1 Cells, But Not Rat Cortical Membranes Possess a Single Population of Receptors

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SUMMARY

Recent work has demonstrated that α_1-adrenergic receptors are composed of at least two subtypes, termed α_{1a} and α_{1b}. It has been proposed that these subtypes may be linked to distinct second messenger systems. In the current studies, we have compared the properties of α_1-adrenergic receptors in rat renal cortical membranes with those in MDCK-D1 cells, a clonal cell line derived from distal tubule/collecting duct. Competitive binding studies with [^{3}H]prazosin and compounds [5-methylurapidil, (+)-niguldipine, WB4101, and oxymetazoline] that distinguish high affinity (α_{1a}) and low affinity (α_{1b}) sites indicated that rat renal cortical membranes contain about 50% of each class of site. In contrast, MDCK-D1 cells contained a single population of low affinity sites. 5-Methylurapidil, but not the other compounds, recognized binding sites in these cells with a substantially lower affinity than has been observed for the low affinity site in other tissues and in parallel studies with renal cortical membranes. [^{3}H]Prazosin binding sites in these cells, as well as α_1-adrenergic receptor-mediated arachidonic acid release and phosphoinositide and phosphatidylcholine hydrolysis, were sensitive to inactivation by chloroethylclonidine (IC_{50} ~ 0.7 μM), as expected for α_{1b} receptors. However, α_1-adrenergic receptors of MDCK-D1 cells required extracellular calcium for biological response, unlike what has been hypothesized for the α_{1b} receptor subtype. These data indicate that the population of α_1-adrenergic receptors of distal tubule/collecting duct cells likely consists of receptors of the α_{1b} subtype. The low affinity binding of 5-methylurapidil and the requirement for extracellular calcium for biological response in these cells suggest that this receptor may not be identical to the α_{1b} receptor that has been observed in other systems.

α_1-Adrenergic receptors mediate a wide variety of physiological responses (for review see Refs. 1–3). It has recently become apparent from pharmacological and functional studies and through utilization of molecular cloning techniques that at least two distinct subtypes of the α_1 receptor are involved in these processes. Competitive antagonists [e.g., WB4101 (1, 4, 5) and 5-methyl urapidil (6–9)], agonists [e.g., oxymetazoline (10, 11)] , and the antihypertensive agent (+)-niguldipine (8, 9, 12) distinguish a high affinity site, α_{1a}, from a low affinity site, α_{1b}, when tested in studies of radioligand binding and/or physiological responses. Moreover, only the α_{1b} subtype is sensitive to inactivation by the alkylating agent CEC (10, 13, 14) and appears to be selectively labeled with the photoaffinity probe ^{125}I-azidoprazosin (15).

Cotecchia *et al.* (16) and Schwinn *et al.* (17) have been able to distinguish between two α_1-adrenergic receptor subtypes by molecular cloning and expression of the receptors. Cotecchia *et al.* (16) ascertained, from hamster smooth muscle cells, the cDNA and amino acid sequences of an α_{1b} receptor, based on affinity of the receptor for various compounds in radioligand binding studies. Schwinn *et al.* (17) recently cloned, from bovine brain, a cDNA that appears to encode a novel subtype, based on sensitivity of the receptor to inactivation by CEC and high affinities for α_{1a}-selective compounds in radioligand binding studies.

Hypotheses have been put forward on how the α_1-adrenergic receptor subtypes may be linked to different signal transduction pathways. The α_{1a} subtype has been proposed to control influx of extracellular Ca^{2+} into the cell through a voltage-dependent channel (7, 14, 18), whereas the α_{1b} subtype mediates hydrolysis of PIP_2 to IP_3 and DAG, through activation of an as yet unidentified GTP-binding protein. IP_3 and DAG act as second messengers, the former mobilizing intracellular Ca^{2+} and the latter activating protein kinase C (9, 18). Tsujimoto *et al.* (14),

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ABBREVIATIONS: CEC, chloroethylclonidine; AA, arachidonic acid and metabolites; PI, phosphoinositide; PIP_2, phosphatidylinositol bisphosphate; IP_3, inositol trisphosphate; PC, phosphatidylcholine; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-pi-perazineethanesulfonate; MDCK-D1, D1 clone of Madin Darby canine kidney cell line; EGTA, ethylene glycol-bis(β-amino-ethyl ether)-*N,N,N′,N′*-tetraacetic acid; IC_{50}, inhibition constant at which 50% of receptors are blocked or 50% of response is inhibited; DAG, diacylglycerol.

however, observed IP_3 formation subsequent to both α_{1a} and α_{1b} subtype activation, a finding recently confirmed by Han *et al.* (19), who suggested that the IP_3 formed might not be the same product from identical precursors for both subtypes. Thus far, limited data have been available regarding linkage of α_1 receptor subtypes to other biochemical responses known to be mediated by the α_1-adrenergic receptor, such as AA release, cAMP and cGMP accumulation, and PC hydrolysis (1–3, 13, 20–22). One means to assess α_1-adrenergic receptor subtypes, which has not been previously tested, is examination of the expression and function of receptor subtypes in cultured cell systems.

In Madin Darby canine kidney cells, a well differentiated renal cell line with properties of the distal tubule/collecting duct (23), distinct responses to α_1-adrenergic receptor activation have previously been well defined. Work in our laboratory has suggested that hydrolysis of PIP_2 by phospholipase C occurs parallel to and independent of mobilization of AA, which likely results from activation of phospholipase A_2 (20). AA release takes place subsequent to protein kinase C activation by DAG, which appears to be formed, at least in part, from hydrolysis of PC (21).

In the current studies, we have compared properties of the α_1-adrenergic receptors of MDCK-D1 cells with those of rat renal cortical membranes, in order to (i) define the nature of the α_1-adrenergic receptors in distal tubule/collecting duct, (ii) determine whether distinct signal transduction pathways are mediated by different α_1-adrenergic receptor subtypes, and (iii) ascertain which subtype(s) is (are) linked to these events.

Experimental Procedures

Materials. [7-*methoxy*-^{3}H]Prazosin (75–87 Ci/mmol), [*methyl*-^{3}H] choline chloride (87 Ci/mmol), and [^{3}H]AA (76–100 Ci/mmol) were purchased from New England Nuclear (Boston, MA). *myo*-[^{3}H]Inositol was purchased from American Radiolabeled Chemicals Inc., St. Louis, MO. WB4101 and CEC were from Research Biochemicals Inc. (Natick, MA). 5-Methylurapidil and (+)-niguldipine were a gift from Byk Gulden Pharmazeutika (Konstanz, FRG). 5-Methylurapidil was also purchased from Research Biochemicals Inc. Phentolamine mesylate was a gift from Ciba-Geigy (Summit, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Cells were cultured as previously described (20, 21). Briefly, MDCK-D1 cells were grown in DMEM supplemented with 10% heat-inactivated serum (7.5% horse serum and 2.5% fetal calf serum), 15 mM HEPES, and 3.7 g/liter sodium bicarbonate, pH 7.4. Subconfluent cells were subcultured with a trypsin/EDTA solution every 3 days. Subconfluent cells cultured in 35-mm (six wells) or 150-mm dishes were used for experiments at day 3 of culture.

Membrane preparations from MDCK-D1 cells and rat kidney cortex. Membranes from MDCK-D1 cells were prepared essentially as previously described (24). Briefly, subconfluent cells in 150-mm culture dishes were washed twice with 5 ml of ice-cold lysis buffer (5 mM Tris·HCl, 2 mM $MgCl_2$, 0.2 mM EGTA, pH 7.5) and incubated with 10 ml of the same buffer at 4° for 10 min. Lysed cells were then harvested using a cell scraper, and dishes were washed once more with 5 ml of lysis buffer, resulting in a pooled lysate of 15 ml/150-mm dish. Pooled lysates were centrifuged at 30,000 × *g* for 10 min at 4°, and pellets were resuspended in 10–15 ml of lysis buffer and homogenized with 20 strokes in a Dounce homogenizer. After this procedure was repeated once more (total of three × 10-min centrifugations), the pellet was resuspended in the appropriate volume (typically pooled lysate from eight 150-mm dishes/5.5 ml) of incubation buffer (50 mM Tris free base, 0.5 mM EDTA free acid, pH 7.5) and used for experiments after homogenization. Protein content/sample was determined according to the method of Lowry *et al.* (25).

Renal cortical membranes were prepared from male Sprague Dawley rats (300–350 g), which were anesthesized with a mixture of 7.5 mg/100 g of body weight ketamine and 0.5 mg/100 g of body weight xylazine. Kidneys were rapidly removed, rinsed in 0.9% NaCl, and dissected to remove medullae. Renal cortices were then frozen in liquid nitrogen and stored at −70° until further use. Membranes from kidney cortex were prepared by thawing of tissue in 10 ml of ice-cold homogenization buffer (20 mM $NaHCO_3$, pH 7.5). After the kidney cortices were minced with scissors, tissues were homogenized with a Tekmar Tissumizer, once for 10 sec at full speed and twice for 20 sec at 25% speed. After centrifugation at 30,000 × *g* for 30 min, the pellet was resuspended in 10 ml of homogenization buffer by pipetting. After another identical centrifugation, the pellet was resuspended in the appropriate volume of incubation buffer (typically 1 g of kidney cortex/17 ml) and homogenized for 10 sec at full speed. This procedure resulted in 250–350 μg of protein/100-μl sample, for use in radioligand binding assays.

Radioligand binding assay with membranes. Assays were performed in duplicate by incubation of 200 μl of [7-*methoxy*-^{3}H]prazosin with 100 μl of membranes, in a final volume of 1 ml of incubation buffer, in 100- × 15.7-mm polypropylene test tubes. Nonspecific binding was determined in the presence of 10 μM phentolamine, and specific binding was defined by subtraction of nonspecific from total binding. Competitors were added as 200-μl aliquots dissolved in incubation buffer for all drugs except (+)-niguldipine. (+)-Niguldipine was added in 10-μl aliquots dissolved in dimethyl sulfoxide, diluted in glass tubes to a final volume of 1 ml, to prevent artifacts caused by nonspecific adherence of this compound as reported by Boer *et al.* (12). Assays were initiated by addition of 100 μl of membranes, containing 75–300 μg/sample, depending on confluency of the cells. For assays done with (+)-niguldipine, samples containing no more than 75–90 μg of protein/100 μl were used, because highly concentrated membrane preparations have been reported (12) to affect pK_I values, due to the fact that (+)-niguldipine is highly lipophilic. We confirmed these results (data not shown). After incubation for 30 min at 37° in a shaking water bath, binding reactions were terminated by rapid filtration over Whatman GF/C filter paper, using a Brandel cell harvester. Tubes were washed twice with 10 ml of incubation buffer, at room temperature, and bound radioligand retained on the filter was counted in a scintillation counter, after addition of 4.5 ml of liquid scintillation fluid.

Radioligand binding assay with MDCK-D1 whole cells. After CEC treatment (see below), cells grown in 35-mm dishes were incubated with appropriate concentrations of [7-*methoxy*-^{3}H]prazosin, in 1 ml of assay medium (DMEM supplemented with 15 mM HEPES, 0.05% bovine serum albumin, pH 7.4). Nonspecific binding was determined by addition of 10 μM phentolamine, and specific binding was calculated by subtraction of nonspecific from total binding. After incubation for 60 min at 37°, cells were washed three times with 1 ml of assay medium. Cells were dissolved in 0.5 ml of 5% sodium dodecyl sulfate, and dishes were rinsed with 0.5 ml of H_2O. Triplicate samples were counted in a liquid scintillation counter.

CEC treatment. On day 3 of culture, medium was aspirated and cells were incubated with appropriate concentrations of CEC, in 1 ml of assay medium/dish. After four washes with 2 ml of assay medium, cells were used for various experiments. [In studies of PI hydrolysis, 50 mM LiCl was added to the assay medium used for washes (20).]

AA release. [^{3}H]AA release was measured as described previously (22). Briefly, cells were subcultured in 35-mm dishes and labeled with 0.30–0.33 μCi/ml/dish [^{3}H]arachidonic acid, in cell culture medium containing only 0.5% fetal calf serum, for 4 hr. Medium was aspirated and cells were incubated with appropriate concentrations of competitors in triplicate, in the absence or presence of 1 μM (−)-epinephrine in assay medium [0.1 mM ascorbic acid was included in freshly prepared (−)-epinephrine stock solutions for all assays, to prevent drug oxidation before addition to the cells]. After incubation at 37° for 30 min, samples of 850 μl were taken, 4.5 ml of liquid scintillation fluid were added, and

samples were counted in a liquid scintillation counter. When cells were treated with CEC (see above), they were washed four times with 2 ml of assay medium before incubation, with or without 1 μM (−)-epinephrine.

PI hydrolysis. Assays were carried out essentially as described previously (26). Briefly, cells cultured in 35-mm dishes were labeled with *myo*-[^{3}H]inositol, 2 μCi in 2 ml of culture medium/dish, for 48 hr. After labeling, cells were either incubated with CEC or treated with 5-methylurapidil, in the absence or presence of 1 μM epinephrine. Washes were done with DMEM supplemented with 15 mM HEPES, 0.05% bovine serum albumin, and 50 mM LiCl, pH 7.4. For the incubation, 1 ml of the same medium was added, with or without 1 μM epinephrine, for CEC-treated cells or medium with 5-methylurapidil, with or without 1 μM epinephrine, and cells were incubated for 30 min at 37°. Reactions were terminated by aspiration of the medium, placement of the dishes on ice, and addition of 1 ml of ice-cold methanol. Cells were then harvested using a cell scraper, and dishes were washed with 1 ml of H_2O/dish. Two milliliters of chloroform were added to samples and, after vortexing, samples were centrifuged for 5 min at 1500 rpm or left for >1 hr at room temperature, to separate the aqueous from the organic phase. Separation of free inositol from inositol phosphates was performed using columns of 300 mg of Dowex (AG 1-x8). Aqueous phases of samples were applied to columns, and free inositol was removed by two washes with 10 ml of H_2O. Inositol phosphates were eluted with 2 × 5 ml of 2.0 M ammonium formate, 100 mM formic acid. Ten milliliters of scintillation fluid were added, and samples were counted in a scintillation counter.

PC hydrolysis. MDCK-D1 cells subcultured in 35-mm dishes were prelabeled with [*methyl*-^{3}H]choline chloride, 4 μCi in 2 ml/dish, for 18–24 hr. As described before (20), these conditions result in about 55% of the label becoming associated with the cells, with 70% of the cell-associated label being present in phospholipids, of which 96% in PC. Cells were washed twice with 2 ml of assay medium and incubated with appropriate concentrations of 5-methylurapidil, in the absence or presence of 1 μM (−)-epinephrine. When cells were treated with CEC, they were washed only after CEC treatment, before incubation with or without 1 μM (−)-epinephrine. After a 30-min incubation at 37°, samples of 850 μl were taken and counted in a liquid scintillation counter, after addition of 4.5 ml of liquid scintillation fluid.

Assays in the absence of extracellular calcium. Solutions were made in plastic containers rinsed with 10 mM EGTA. In assay medium, $CaCl_2$ was replaced by 1 mM $MgCl_2$. Cells were incubated for only 20 min, to prevent artifacts caused by changes in viability of the cells over time under these conditions.

Data analysis. Samples were counted for radioactivity in a Beckman liquid scintillation counter (efficiency, 42%). For functional assays, control values obtained by incubation in the absence of (−)-epinephrine were subtracted from values obtained after stimulation with (−)-epinephrine. All curves were fit to data using Graphpad InPlot for nonlinear regression to appropriate equations. Dissociation constants (K_D) and total numbers of receptors (B_{max}) were determined by saturation isotherms, generated with Graphpad InPlot. All data are expressed as the mean ± standard error of the mean of three experiments, unless noted otherwise in figure or table legends.

Results

MDCK-D1 cells contain a homogeneous population of α_1-adrenergic receptors, in contrast to kidney cortex, which contains two α_1-adrenergic receptor subtypes. When 5-methylurapidil, (+)-niguldipine, WB4101, and oxymetazoline were used to compete for [^{3}H]prazosin binding to rat renal cortical membranes, data from experiments with all four competitors were best fit to a two-site model (Fig. 1). The shallow curves obtained imply the existence of two binding sites, one with high and one with low affinity for these compounds. All of the competitors tested have been used to distinguish α_{1a} (high affinity) and α_{1b} (low affinity) receptor binding sites in other tissues (1, 4–12). Table 1 shows $-\log(K_I)$ values for both sites, as well as the relative population of each site found with the four different competitors. All four compounds showed approximately equal populations of high and low affinity binding sites. $-\text{Log}(K_I)$ values are in good agreement with values published before for affinities of these compounds for the α_{1a}- (high affinity) and α_{1b}- (low affinity) adrenergic receptor subtypes (4–12). Of all the compounds, (+)-niguldipine differentiated most effectively between high and low affinity binding sites, i.e., showed the greatest difference between $\log(K_I)$ values for the two binding sites.

In contrast, when membrane preparations of MDCK-D1 cells were tested with the same compounds in [^{3}H]prazosin binding studies, we obtained strikingly different results. With all compounds, we found steep monophasic competition curves, compatible with the existence of a single subtype of receptor (Fig. 1). As shown in Table 1, $-\log(K_I)$ values yielded only a low affinity site in studies with MDCK-D1 membranes. Affinities of (+)-niguldipine, WB4101, and oxymetazoline were in good agreement with affinities found for the low affinity site in renal cortical membranes. 5-Methylurapidil, however, had >6 times lower affinity in the MDCK-D1 cell, in comparison with kidney cortex ($p < 0.0009$, by one-tailed unpaired t test).

Although 5-methylurapidil is reported to be a competitive antagonist in various tissues (7), we investigated the hypothesis that the much lower affinity for the receptor in MDCK-D1 membranes, compared with the affinity for the low affinity site in renal cortical membranes, might be due to effects of 5-methylurapidil other than those expected for a competitive antagonist. As shown in Fig. 2, competition of 5-methylurapidil for various concentrations of [^{3}H]prazosin resulted in an increase in K_D, without affecting B_{max} (Table 2), indicating that in MDCK-D1 cells the binding pattern for 5-methylurapidil is that of a competitive antagonist.

Taken together, the binding data demonstrate the existence of high (α_{1a}) and low (α_{1b}) affinity binding sites in approximately equal abundance in rat renal cortical membranes. In MDCK-D1 membranes, only a single class of low affinity sites could be detected, which showed a substantially lower affinity for 5-methylurapidil than did the low affinity sites in renal cortical membranes.

CEC treatment inhibits receptor binding, PI and PC hydrolysis, and AA release in MDCK-D1 cells, with similar concentration dependence. Because CEC has been proposed as an agent that selectively inhibits α_{1b} receptor subtypes (10, 13, 14), we investigated the effects of CEC on receptor binding and various α_1-adrenergic receptor-mediated responses in MDCK-D1 cells. We initially performed experiments to test the specificity of CEC for the receptor and to determine the optimal conditions for the alkylating effect of CEC in MDCK-D1 cells. Incubation of cells with 3 μM CEC for 30 min and then washing of cells to remove unbound CEC substantially decreased (−)-epinephrine- but not bradykinin- and ATP-promoted AA release (data not shown). Thus, CEC treatment selectively blocked the α_1-adrenergic receptor-mediated response. In addition, cells that had been treated with CEC were optimally responsive when incubated with (−)-epinephrine for 30 min. When incubated in the absence of CEC for 60 min, cells failed to recover responses that had been lost after a 30-min CEC treatment. Because longer incubation times

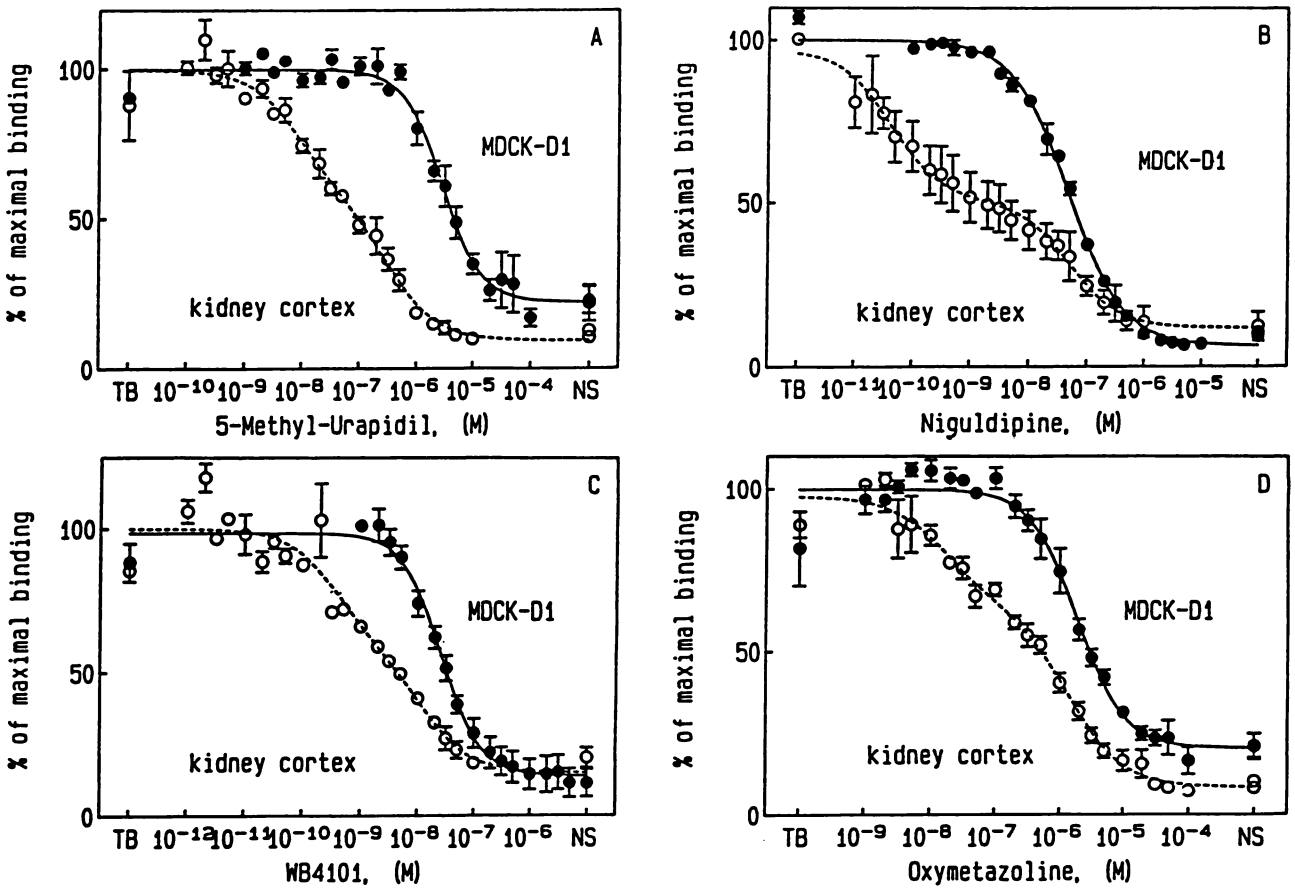

Fig. 1. Competition of 5-methylurapidil (A), (+)-niguldipine (B), WB4101 (C), and oxymetazoline (D) for [^{3}H]prazosin binding (0.2–0.4 nM) in membranes from kidney cortex (○) and MDCK-D1 cells (●). Experiments were performed as described in Experimental Procedures. Each *point* is the average of the number of experiments given in Table 1, performed in duplicate, ± standard error of the mean. *TB*, total binding; *NS*, nonspecific binding.

TABLE 1

Affinity of various competitors for [^{3}H]prazosin binding sites in membranes from kidney cortex and MDCK-D1 cells

$-\text{Log}(K_i)$ values were calculated using Graphpad InPlot, after competition curves were generated using nonlinear regression to a two-site model (kidney cortex) or to a one-site model (sigmoid curve; MDCK-D1 cells). Values are means ± standard errors of the mean from three experiments, except for 5-methylurapidil, which was tested in four experiments with renal cortical membranes, and WB4101, which was tested in four or two experiments in membranes of kidney cortex or MDCK-D1 cells, respectively.

	$-\text{Log}(K_{i_{high}})$ (% of sites)	$-\text{Log}(K_{i_{low}})$ (% of sites)
Kidney cortex		
(+)-Niguldipine	10.88 ± 0.24 (56 ± 5%)	7.79 ± 0.32 (44 ± 5%)
5-Methylurapidil	8.61 ± 0.15 (45 ± 5%)	6.96 ± 0.10 (55 ± 5%)
WB4101	9.86 ± 0.22 (51 ± 9%)	8.29 ± 0.09 (49 ± 9%)
Oxymetazoline	8.52 ± 0.19 (39 ± 9%)	6.43 ± 0.03 (61 ± 4%)
MDCK-D1		
(+)-Niguldipine		7.95 ± 0.02
5-Methylurapidil		6.17 ± 0.03
WB4101		8.20 ± 0.09
Oxymetazoline		6.42 ± 0.08

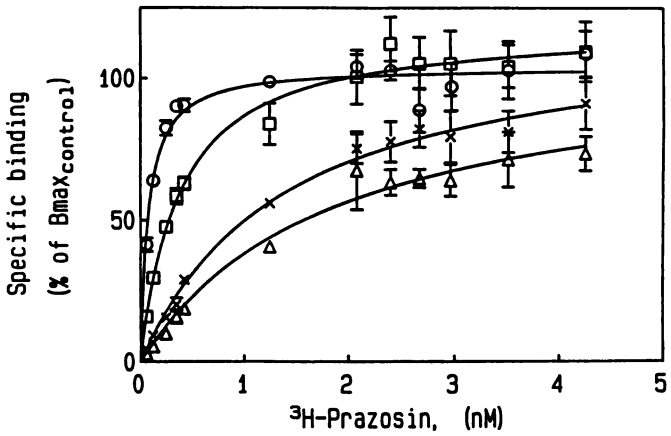

Fig. 2. Saturation isotherms of [^{3}H]prazosin binding to membranes of MDCK-D1 cells in the absence or presence of 5-methylurapidil. ○, Control; □, 1 μM 5-methylurapidil; ×, 6 μM 5-methylurapidil; △, 10 μM 5-methylurapidil. Each *point* is expressed as a percentage of the total number of receptors found when the experiment was performed in the absence of 5-methylurapidil (control). Concentrations of [^{3}H]prazosin from averaged experiments deviated ≤10%.

did not produce greater inhibition of response, we chose 30 min as the optimal period for treatment of cells with CEC.

When cells were treated with various concentrations of CEC, 50% of the receptors were inactivated at a concentration of 0.7 μM CEC (Fig. 3). In order to see whether we could find a correspondence between loss of receptors and inhibition of α_1-adrenergic receptor-mediated responses to the receptor, we tested PI and PC hydrolysis and AA release after pretreatment of cells with CEC. As shown in Fig. 3, all three responses were sensitive to CEC over a similar concentration range. The $-\log(IC_{50})$ value for receptor binding was 6.18 ± 0.13 (two experiments), for AA release, 6.24 ± 0.18, and for PI hydrolysis (two experiments) and PC-hydrolysis, 5.87 ± 0.02 and 6.39 ±

TABLE 2

Dissociation constants (K_D) and total number of receptors (B_{max}) derived from saturation isotherms of [^{3}H]prazosin binding to membranes of MDCK-D1 cells, in the absence and presence of 5-methylurapidil (5-MU)

	K_D	B_{max}
	nM	% of control
Control	0.076	100
1 μM 5-MU	0.376	114
6 μM 5-MU	1.421	116
10 μM 5-MU	1.864	105

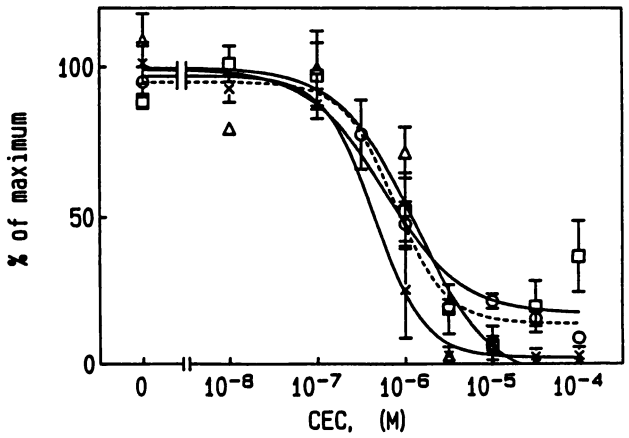

Fig. 3. Inhibition of specific [^{3}H]prazosin (0.3 nM) binding (O – – – O) and (−)-epinephrine-stimulated AA release (□), PI hydrolysis (△), and PC hydrolysis (×) by CEC in MDCK-D1 cells. Experiments were performed as described in Experimental Procedures.

0.19, respectively. Although the pooled data, shown in Fig. 3, provide evidence for loss of receptor binding and response over a similar concentration range of CEC treatment of cells, maximal loss in some experiments was only about 80%. In other experiments, all responses or receptor binding were lost. We do not know the reason for this variability, but it might be related to inaccessibility of receptors located on the basolateral membranes of these polarized epithelial cells. Together, these data suggest that a single class of α_1-adrenergic receptors, which is CEC sensitive, mediates all the pathways thus far identified as being involved in signal transduction in MDCK-D1 cells.

5-Methylurapidil competes with very low affinity for [^{3}H]prazosin receptor binding and, correspondingly, high concentrations of 5-methylurapidil are required to inhibit (−)-epinephrine-promoted PI and PC hydrolysis and AA release in MDCK-D1 cells. To investigate whether the low affinity of 5-methylurapidil for the α_1 receptors in MDCK-D1 cells (Fig. 2) was relevant to the antagonizing activity of the compound on functional events, we tested the inhibitory effect of varying concentrations of 5-methylurapidil on (−)-epinephrine-promoted PI and PC hydrolysis and AA release. We found that a relatively high concentration of 5-methylurapidil was necessary to inhibit PI hydrolysis [−log(IC_{50}), 5.83 ± 0.16], PC hydrolysis (6.21 ± 0.09), and AA release (5.5 ± 0.09), similar to what was found for competition for [^{3}H]prazosin binding sites (Fig. 1A).

AA release in MDCK-D1 cells is sensitive to both inactivation of the α_1-adrenergic receptor by CEC and the presence of extracellular Ca^{2+}. Previous studies have suggested that (−)-epinephrine-promoted AA release in MDCK-D1 cells is dependent on extracellular Ca^{2+} (20) but that smooth muscle contraction mediated by the α_{1a} but not by the α_{1b} receptor is dependent on extracellular Ca^{2+} (18). Because MDCK-D1 cells appeared to possess a single population of α_1-adrenergic receptors that resembled α_{1b} receptors in their sensitivity to CEC and in their recognition of several competitive agents, we tested the Ca^{2+} requirement of CEC-sensitive sites in MDCK-D1 cells. Pretreatment of cells with 3 μM CEC caused a 73 ± 21% inhibition of (−)-epinephrine-promoted AA release. Similarly, if AA release was measured when cells were incubated in Ca^{2+}-free medium, a 71 ± 12% inhibition occurred (in the absence of CEC). When both conditions were applied, 90 ± 5% of the response was lost (Fig. 4). These data demonstrate that a functional response (e.g., AA release), mediated by the homogenous population of α_1-adrenergic receptors in MDCK-D1 cells, can be both sensitive to CEC and dependent on the presence of extracellular Ca^{2+}. This pattern appears to be atypical for either the α_{1a}- or the α_{1b}-site that have been described previously (18, 19).

Discussion

In the current work, radioligand binding studies showed that MDCK-D1 cells, renal epithelial cells derived from distal tubule/collecting duct, contain a single population of α_1-adrenergic receptors. In contrast, studies with rat renal cortical membranes indicate the existence of two different subtypes of the α_1 receptor. Competition of (+)-niguldipine, 5-methylurapidil, WB4101, and oxymetazoline for [^{3}H]prazosin binding distinguished a high affinity site, α_{1a}, from a low affinity site, α_{1b}. Data obtained with those compounds suggest an almost equal population of α_{1a} (47 ± 10%) and α_{1b} sites (53 ± 10%) in rat kidney cortex. This estimate is similar to that reported by Han *et al.* (19) for collagenase-dispersed rat renal cells (which are thought to be predominantly tubular cells), in which a 60:40 ratio was found for α_{1a} and α_{1b} subtypes, based on CEC inactivation of and WB4101 competition for radioligand binding sites. α_1 receptors in MDCK-D1 cells had low affinity for all four tested compounds, with a remarkably low affinity for 5-methylurapidil. In the MDCK-D1 cell line, >6 times the concentration of 5-methylurapidil that was needed to occupy 50% of the receptors was necessary to occupy 50% of the α_{1b} popu-

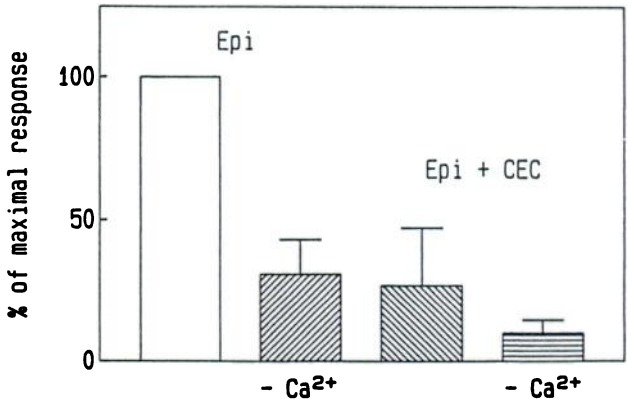

Fig. 4. Effect of CEC and/or the presence of extracellular Ca^{2+} on (−)-epinephrine-stimulated AA release. Cells were incubated with or without 1 μM (−)-epinephrine (*Epi*) under normal conditions, after pretreatment with CEC (3 μM), in the absence of extracellular Ca^{2+}, or under both conditions (CEC pretreatment and in the absence of extracellular Ca^{2+}). Data are expressed as a percentage of the response obtained under normal conditions.

lation of rat kidney cortex (Fig. 1, Table 1). Moreover, if the affinity of 5-methylurapidil for the α_1 receptor in MDCK-D1 cells is compared with affinities for the α_{1b} subtype in various rat tissues (Table 3), the log(K_I) value found with MDCK-D1 cells is >10 times lower than the average log(K_I) values found in those tissues (Table 3).

Further evidence for the existence of a homogenous population of α_1-adrenergic receptors in MDCK-D1 cells was provided by the similar concentration dependence for inhibition of radioligand receptor binding and α_1-adrenergic receptor-mediated functional responses (PI and PC hydrolysis and AA release), by both the alkylating agent CEC (Fig. 3, Table 3) and the competitive antagonist 5-methylurapidil.

In efforts to attribute signal transduction pathways to distinct α_1-adrenergic receptor subtypes, it has been hypothesized that the α_{1a} and the α_{1b} subtypes might control intracellular Ca^{2+} levels in different ways, with the α_{1a} subtype controlling influx of extracellular calcium through voltage-sensitive channels and the α_{1b} subtype mobilizing intracellular calcium by the second messenger IP_3 (18). Han *et al.* (18) reported that in spleen, shown to contain only the α_{1b} subtype, contractile responses were independent of the presence of extracellular calcium, whereas in vas deferens, which contains both α_{1a} and α_{1b} subtypes (40 and 60%, respectively), contractile responses were markedly reduced when Ca^{2+} was removed from the bathing fluid and 0.1 mM EGTA was added. From these data, it was hypothesized that dependence on extracellular calcium might provide a means of differentiating between α_{1a}- and α_{1b}-adrenergic receptor subtypes. In MDCK-D1 cells we found that a single population of receptors, which have α_{1b}-like low affinity for various (ant)agonists in radioligand binding studies, mediate responses that are both sensitive to CEC and dependent on extracellular calcium. This is true for AA release (Fig. 5) and for PC hydrolysis.[1] These data contradict the hypothesis that extracellular calcium dependence provides a clear-cut way to distinguish α_{1a}- and α_{1b}-adrenergic receptor subtypes.

Another explanation for the unconventional properties that we found for the α_1-adrenergic receptor in MDCK-D1 cells could be the existence of another, as yet unidentified, subtype of the α_1-adrenergic receptor in these cells. Because MDCK-D1 cells are cells grown in long term culture, we cannot exclude the possibility that these cells have evolved an α_1-adrenergic receptor that is different from the receptors of tubular cells *in vivo*. Another possibility is that in MDCK cells a "standard" α_{1b} receptor is linked to one or more GTP-binding proteins that are different from those in rat kidney cortex. Because the ligands we used to characterize binding are all antagonists, we think this is an unlikely possibility. An additional possibility is that we are observing a species difference between rat (Table 3) and dog.

Schwinn *et al.* (17) recently reported an α_1-adrenergic receptor from bovine brain, which is sensitive to inactivation by CEC and has an amino acid sequence that is 72% identical (within membrane-spanning domains) to the sequence for the α_{1b} subtype. However, high affinities of the receptor for WB4101, oxymetazoline, and phentolamine were found in ^{125}I-HEAT (2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)-ethylaminomethyl]tetralone) binding studies, similar to affinities described in various systems as being characteristic of the α_{1a} subtype. Thus, this site does not appear to be identical to the site that we have observed. These data, and the data we present in this paper, strongly suggest that subclassification of the α_1-adrenergic receptor will involve more than only the two subtypes (α_{1a} and α_{1b}) that have been described to date.

In this paper we have shown that MDCK-D1 cells contain a single population of α_1-adrenergic receptors, which are able to mediate distinct signal transduction pathways, PIP_2 hydrolysis, AA release, and PC hydrolysis. The identity of this population remains to be established, but it may represent a variant of the α_{1b}-adrenergic receptor subtype.

TABLE 3

−Log(K_I) values of 5-methylurapidil competing for [^{3}H]prazosin binding to low affinity (α_{1b}) α_1-adrenergic receptors in various rat tissues (6–9), compared with those values obtained with MDCK-D1 cells

Values were averaged when more than one reference was available for the same tissue.

	−Log(K_I)
Hippocampus	7.13 ± 0.07
Cerebral cortex	7.37 ± 0.02
Vas deferens	7.60 ± 0.19
Heart	7.41 ± 0.02
Liver	7.64 ± 0.03
Spleen	7.54 ± 0.07
Mean	7.46
MDCK-D1	6.17 ± 0.03

[1] Unpublished observations.

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