MOLECULAR PHARMACOLOGY, 22:532-546

Characterization of *Alpha*-Adrenergic Receptor Subtypes in the Rat Renal Cortex

Differential Regulation of *Alpha*$_1$- and *Alpha*$_2$-Adrenergic Receptors by Guanyl Nucleotides and Na^+

MARSHALL D. SNAVELY AND PAUL A. INSEL

Division of Pharmacology, Department of Medicine, M-013 H, University of California, San Diego, La Jolla, California 92093

Received December 7, 1982; Accepted June 8, 1982

SUMMARY

We have used the subtype-selective radioligands [^{3}H]prazosin (an *alpha*$_1$-adrenergic antagonist) and [^{3}H]yohimbine (an *alpha*$_2$-adrenergic antagonist) to examine *alpha*-adrenergic receptors in rat renal cortical membranes. Under the conditions used in this study, [^{3}H]prazosin bound only to *alpha*$_1$-adrenergic receptors, whereas [^{3}H]yohimbine bound only to *alpha*$_2$-adrenergic receptors; the two radioligands were completely selective and did not bind to a common site. The ratio of *alpha*$_2$- to *alpha*$_1$-adrenergic receptors was about 3:1. Guanyl nucleotides decreased the affinity of epinephrine at both receptor subtypes, but this effect was greater at the *alpha*$_2$-recepter and, according to computer analysis, occurred through different mechanisms at the two receptor subtypes. NaCl decreased the affinity of epinephrine at both *alpha*-receptor subtypes; this effect was more Na^+-selective at *alpha*$_2$- than at *alpha*$_1$-receptors. Guanyl nucleotides and NaCl were additive in decreasing the affinity of epinephrine at the *alpha*$_1$-receptor but were synergistic at the *alpha*$_2$-receptor. In addition, NaCl increased specific binding of [^{3}H] yohimbine but had no effect on the binding of [^{3}H]prazosin. This enhancement of [^{3}H] yohimbine binding was Na^+-specific and fully reversible, and represented an increase in maximal binding capacity. Although binding of epinephrine to both *alpha*$_1$- and *alpha*$_2$-receptors could be modulated by guanyl nucleotides, we were unable to detect inhibition by epinephrine of basal or hormone-stimulated adenylate cyclase activity. Thus, separate *alpha*$_1$- and *alpha*$_2$-adrenergic receptors can be detected in the rat renal cortex and binding to both receptor subtypes can be regulated by guanyl nucleotides and Na^+. Na^+ may directly interact with *alpha*$_2$- but not *alpha*$_1$-adrenergic receptors in the renal cortex. Our findings with renal cortical membranes indicate that regulation of agonist binding at *alpha*-adrenergic receptor subtypes by guanyl nucleotides and Na^+ is not limited to *alpha*$_2$-adrenergic receptors, as previously reported with other tissues.

INTRODUCTION

Catecholamines, in particular those acting at *alpha*-adrenergic receptors, are important regulators of renal function (reviewed in refs. 1 and 2). *Alpha*$_1$-adrenergic receptors stimulate renal gluconeogenesis and regulate renal vascular tone, and other *alpha*-adrenergic receptors inhibit renin release and modulate reabsorption of sodium and water in the proximal tubule. In spite of the role of *alpha*-adrenergic receptors in regulating renal function, no detailed biochemical characterization of these receptors has been reported. Although several preliminary studies of radioligand binding to renal *alpha*-adrenergic receptors have appeared (3–7), these studies have failed to define adequately the *alpha*-adrenergic receptor subtypes in kidney tissue. Moreover, no previous information has been available regarding the regulation of these receptors by nucleotides and ions, which modulate binding at *alpha*-adrenergic receptors in other tissues (8). Because of its critical role in transporting, concentrating, and excreting ions—in particular, monovalent cations and anions—the kidney is an important tissue in which to explore regulation of receptors by these ions.

Recently, two ligands have been used to label *alpha*$_1$- or *alpha*$_2$-adrenergic receptors with a high degree of

This work was supported by Grants HL 25457 and PHS 5T32HL07261-04 from the National Institutes of Health, an American Heart Association Grant-in-Aid (79-680), a National Science Foundation Predoctoral Fellowship (M. D. S.), and an American Heart Association Established Investigatorship (P. A. I.) Portions of this work were presented at the meeting of the American Society of Biological Chemists, St. Louis, Mo., June 3, 1981 [*Fed. Proc.* 40:1823 (1981)].

0026-895X/82/060532-15$02.00/0

selectivity. [^{3}H]Prazosin has been shown to bind to *alpha*$_1$-adrenergic receptors in membranes prepared from heart (9) and liver (10, 11), whereas [^{3}H]yohimbine has been used to identify *alpha*$_2$-receptors in membranes from platelets (12), liver (10), and adipocytes (13). Selective labeling of the two receptor subtypes has demonstrated certain biochemical differences between them. In particular, agonist binding to *alpha*$_1$- and *alpha*$_2$-adrenergic receptors appears to be modulated differently by guanyl nucleotides (8, 10). For these reasons we set out to characterize *alpha*$_1$- and *alpha*$_2$-adrenergic receptors in membranes prepared from rat renal cortex using the *alpha*$_1$-selective antagonist, [^{3}H]prazosin, and the *alpha*$_2$-selective antagonist, [^{3}H]yohimbine. With assays validated for these two radioligands, we examined differences between the two *alpha*-receptor subtypes in their regulation by Na^+ and guanyl nucleotides.

EXPERIMENTAL PROCEDURES

Materials

The following chemicals were received as gifts from the sources indicated: clonidine HCl (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); phentolamine mesylate (Ciba-Geigy Corporation, Summit, N. J.); (−)-epinephrine, (+)-epinephrine, (−)-isoproterenol, and (−)-norepinephrine [all (+)-bitartrate salts from Sterling Winthrop Research Institute, Rensselaer, N. Y.]; (−)-α-methylnorepinephrine [(−)-nordefrin], free base (Sterling Winthrop Research Institute); [^{3}H]prazosin (33 Ci/mmole) and prazosin HCl (Pfizer Laboratories, New York, N. Y.); (−)-propranolol HCl (Ayerst Research Laboratories, New York, N. Y.); indoramin HCl (Wyeth Laboratories, Philadelphia, Pa.). Parathyroid hormone, salmon calcitonin, and glucagon were generous gifts from the laboratories of Drs. M. Geoffrey Rosenfeld, Leonard Deftos, and Gordon Sato (all of whom are located at University of California, San Diego), respectively. The following chemicals were purchased from the sources indicated: (−)-phenylephrine, arginine vasopressin, NaF, and *N*-methyl-D-glucamine (Sigma Chemical Company, St. Louis, Mo.); yohimbine HCl (United States Biochemical Corporation, Cleveland, Ohio); [^{3}H]yohimbine (81 Ci/mmole) (New England Nuclear Corporation; Boston, Mass.). The purity of the radioligands was monitored intermittently by thin-layer chromatography.

Methods

Preparation of renal cortical membranes. Male Sprague-Dawley rats, 5–10 weeks old, were killed by decapitation. The animals were exsanguinated by holding them by their tails in order to minimize contact of the kidneys with agonally released catecholamines. The kidneys were rapidly removed (less than 1 min after decapitation) and placed on ice in sucrose buffer containing 0.32 M sucrose, 5 mM Tris, and 1 mM $MgCl_2$ (pH 7.5). All subsequent steps were conducted at 4°. The kidneys were bissected and the medulla and papilla were dissected free of the cortex and discarded. The remaining cortical tissue was weighed, minced, and homogenized in 20 volumes of sucrose buffer with six strokes of a Potter-Elvejhem Teflon-glass tissue homogenizer. The crude homogenate was filtered through four layers of cheesecloth. The resulting filtrate was centrifuged at 500 × *g* for 5 min and the pellet was discarded. The supernatant was centrifuged at 30,000 × *g* for 25 min. The resulting pellet was resuspended, washed once in incubation buffer, and then resuspended to a protein concentration of 2 mg/ml in the incubation buffer containing 50 mM Tris and 10 mM $MgCl_2$ (pH 7.5). All experiments were conducted with freshly prepared membranes. Electron microscopic examination of these membranes revealed membrane fragments but no vesicles. Protein concentration was determined by the method of Lowry *et al.* (14), using bovine serum albumin standards.

Radioligand binding assay. Binding assays were generally performed in triplicate by incubating 0.150–0.200 ml of membrane suspension with 0.025 ml of radioligand and 0.025–0.075 ml of various drugs, ions, or nucleotides in polypropylene test tubes (16 × 105 mm, Sarstedt). In some experiments conducted with [^{3}H]prazosin, the reaction volume was doubled in order to compensate for the low specific activity of the radioligand. Assays were initiated with the addition of membrane and were carried out in buffer containing 50 mM Tris and 10 mM $MgCl_2$ (pH 7.5 at 25°). For assay of *alpha*$_1$-receptors, membranes were incubated with [^{3}H]prazosin for 50–60 min at 25° in a shaking water bath (120 cycles/min). The reaction was terminated with the addition of 10 ml of the incubation buffer at 25°. The bound and free radioligands were then separated by rapid (<10 sec) filtration over glass-fiber filters (Whatman-GF/C) on a Millipore filtration manifold. The filters were then washed with 10 ml of incubation buffer at 25°. For assay of *alpha*$_2$-receptors, membranes were incubated with [^{3}H]yohimbine for 30 min at 25° in a shaking bath (120 cycles/min). The reaction was terminated with the addition of 10 ml of incubation buffer at 4° to retard the rapid dissociation of [^{3}H]yohimbine from the receptor, and the filters were washed with 10 ml of incubation buffer at 4°.

Filtration and washing for assays of both [^{3}H]prazosin and [^{3}H]yohimbine binding were rapid enough so that less than 5% of the specifically bound radioligand dissociated. The radioactivity retained on the filters was counted at 40% efficiency in plastic minivials containing 4 ml of New England Nuclear Formula 947 liquid scintillation cocktail. Nonspecific binding for both radioligands was defined as the amount of binding measured in the presence of 10 μM phentolamine. This concentration, 400-fold greater than the K_D of phentolamine at both *alpha*-receptor subtypes (Table 1), was chosen because it occupied more than 95% of the binding sites for both radioligands under the conditions used in the study. Subtraction of nonspecific binding from total binding yielded specific binding which was 60–80% for both radioligands at concentrations near their respective K_D values. Specific binding of both radioligands was linear with protein concentration up to 0.5 mg of protein per assay tube. In general, 0.3–0.4 mg of protein per tube was used. Thin-layer chromatography of bound radioligand eluted from filters demonstrated that no breakdown of either radioligand occurred during the course of the assay. When catecholamines were used in binding experiments, freshly prepared ascorbic acid (free acid) was

included at a final concentration of 0.5 mg/ml in order to prevent oxidation. In such cases, ascorbic acid was also added to total and nonspecific binding tubes; the addition of this concentration of ascorbic acid did not alter the K_D or B_{max} of binding of either radioligand.

Adenylate cyclase assay. Adenylate cyclase activity was assayed in a reaction mixture containing the following final concentrations: 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 100 μM GTP, 0.6 mM [α-^{32}P]ATP (1 to 2 × 10^6 cpm), 0.5 mM cyclic AMP, 2 mM mercaptoethanol, 20 mM phosphocreatine, creatine phosphokinase (33 units/ml), bovine serum albumin (0.1 mg/ml), and renal cortical membranes (0.1–0.2 mg of protein). The final volume of the reaction mixture was 0.1 ml. Assays were performed in the presence and absence of 200 mM NaCl and other additions as indicated. As in binding assays, ascorbic acid (0.5 mg/ml final concentration) was used to prevent oxidation of added catecholamines. Assays were initiated with the addition of membrane suspension. Incubation was carried out at 30° for 20 min, and the reaction was terminated with the addition of 0.9 ml of a solution containing 10% sodium dodecyl sulfate, 0.5 mM cyclic AMP, and 5 mM ATP. Approximately 25,000 cpm of cyclic [^{3}H]AMP were added to each tube in order to determine recovery, and all samples were then boiled for 3 min. Following the method of Salomon (15), cyclic AMP was then isolated by sequential chromatography over Dowex and neutral alumina columns. Under the conditions described, the generation of cyclic AMP was linear with both time and protein concentration.

Data analysis. In order to determine the relative affinities of adrenergic agents at sites labeled by [^{3}H]prazosin and [^{3}H]yohimbine, membranes were incubated with radioligand and varying concentrations of the unlabeled competing agent. A competition curve was then constructed which showed the decrease in radioligand binding with increasing concentrations of competitive agent. The K_D of the unlabeled agent was calculated from the EC_{50}, the concentration of this agent which decreased binding of the radioligand by 50%. The equation used was as follows:

$$K_D = \frac{EC_{50}}{1 + (S/K_m)}$$

where K_D is the dissociation constant of the unlabeled agent, S is the concentration of radioligand, and K_m is the dissociation constant of the radioligand (16).

Hill slopes of saturation isotherms for radioligand binding were determined from the slope of a plot of $\log(B/(B_{max} - B))$ versus $\log[L]$ according to the following equation:

$$\log\left(\frac{B}{B_{max} - B}\right) = n \log[L] - \log K_D'$$

For this equation, n is the theoretical number of ligand binding sites per receptor molecule (Hill slope), K_D' is the apparent dissociation constant of the ligand, B_{max} is the maximal number of binding sites determined by Scatchard analysis, $[L]$ is the concentration of radioligand, and B is the amount of radioligand bound at a given concentration.

Pseudo-Hill slopes for agents competing for radioligand binding sites were determined from the slope of a plot of $\log[\%B_{max}/(100 - \%B_{max})]$ versus log[competing agent].

K_D, the dissociation constant, and B_{max}, the maximal number of binding sites, for both radioligands were determined from Scatchard analysis of saturation isotherms. Plots of B/F versus B were constructed, where B is the amount of radioligand specifically bound and F is the free concentration of radioligand in the assay (the amount of radioligand added minus the amount of total binding). The K_D of the binding ligand was calculated by taking the negative reciprocal of the slope of the plot, and the B_{max} was given by the intercept with the abscissa. The line of the Scatchard plot was determined by regression analysis.

Data from competition of (−)-epinephrine for [^{3}H]prazosin and [^{3}H]yohimbine binding sites were analyzed by a computer program that performs iterative nonlinear regression analyses (17). This program fits binding data to equations describing the law of mass action for one and two classes of binding sites and determines whether the fit for the two-site model is statistically better than that for the one-site model. The output includes affinities for competition curves (for one or both sites) as well as the relative proportions of each of the two sites (where applicable). The program also allows simultaneous analysis of more than one competitive curve to obtain combined values for affinities and percentages of sites.

RESULTS

Kinetics of [^{3}H]prazosin and [^{3}H]yohimbine binding. Binding of [^{3}H]prazosin to rat renal cortical membranes was saturable with time, reaching equilibrium within approximately 20 min, and was rapidly reversible (Fig. 1A). Specific binding was stable for 180 min, the longest time tested, and reversal of specific binding was more than 90% complete after 135 min. A plot of $\ln[B_{eq}/(B_{eq} - B_t)]$ (where B_{eq} = binding at equilibrium and B_t = binding at time, t) versus time for association was linear and yielded an apparent association rate, or k_{obs}, of 0.16 min^{-1} (Fig. 1B). The plot of $\ln[B_t/B_{eq}]$ versus time for dissociation likewise produced a straight line, indicating the expected first-order kinetics, with a slope equal to 0.019 min^{-1}. This corresponds to the dissociation rate constant of binding, or k_2 (Fig. 1C). From these two values the bimolecular association rate constant (k_1) and kinetically derived equilibrium dissociation constant (K_D) were calculated with the following equations:

$$k_1 = \frac{k_{obs} - k_2}{[\text{ligand}]} = \frac{0.16\ \text{min}^{-1} - 0.019\ \text{min}^{-1}}{1.5 \times 10^{-9}\ \text{M}}$$

$$= 9.4 \times 10^7\ \text{min}^{-1}\ \text{M}^{-1}$$

$$K_D = \frac{k_2}{k_1} = \frac{0.019\ \text{min}^{-1}}{9.4 \times 10^7\ \text{min}^{-1}\ \text{M}^{-1}} = 2.0 \times 10^{-10}\ \text{M}$$

The binding of [^{3}H]yohimbine to rat renal cortical membranes also was saturable with time and rapidly reversible (Fig. 2A). In this case, the association and dissociation rates were much more rapid than those obtained with [^{3}H]prazosin. Specific [^{3}H]yohimbine binding was stable for up to 60 min, the longest time tested, and total reversal of specific binding was achieved within about 10 min. Plots of association and dissociation

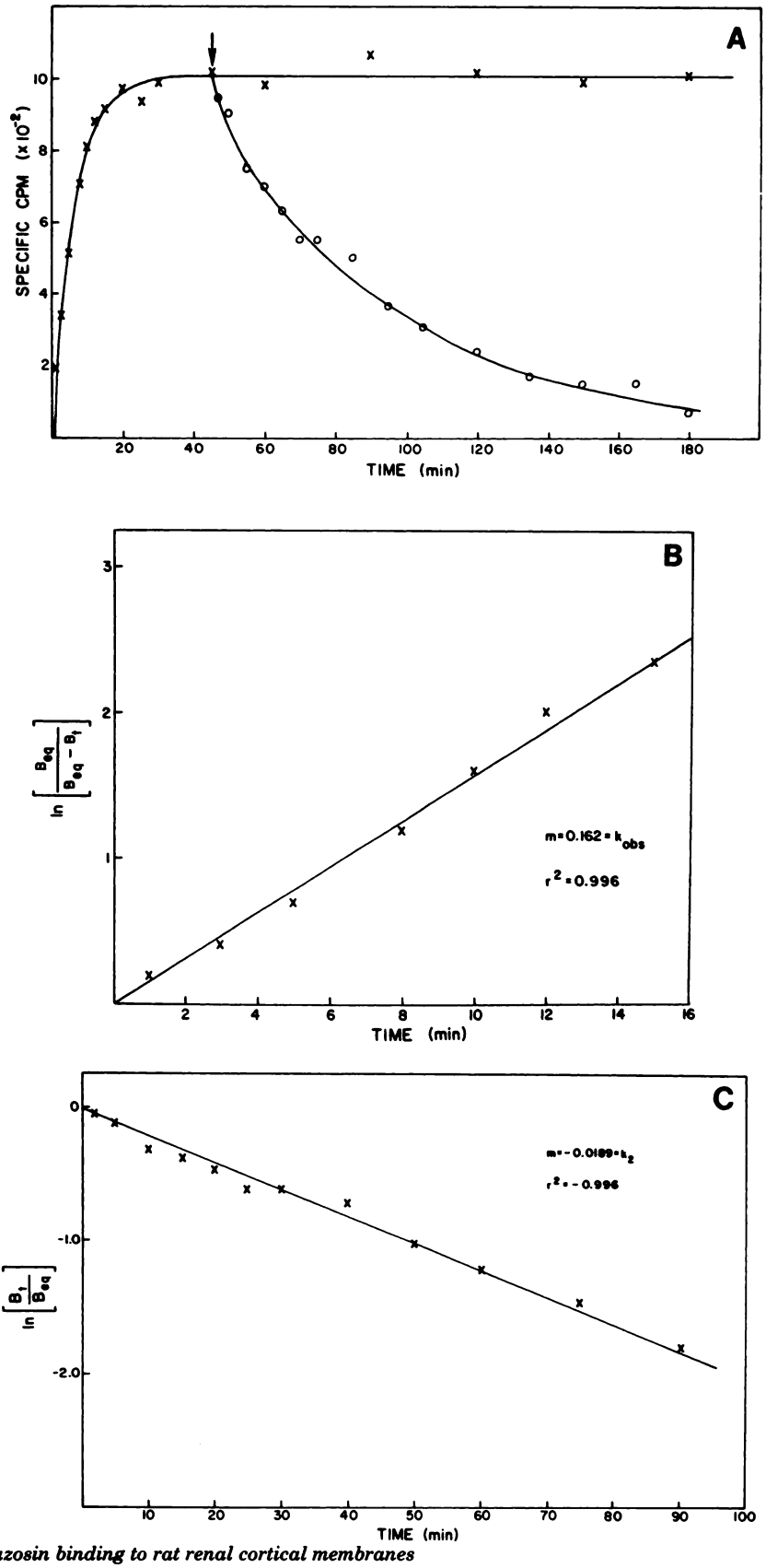

FIG. 1. *Kinetics of [^{3}H]prazosin binding to rat renal cortical membranes*

A. Membranes were prewarmed to 25° for 10 min and incubated batchwise with 1.5 nM [^{3}H]prazosin in two tubes in the absence (total binding) or presence (nonspecific binding) of 10 μM phentolamine. At varying times, duplicate 0.5-ml aliquots were removed from each tube and placed in smaller binding tubes. The reaction was stopped and the samples were filtered and counted as described under Methods. For reversal of binding, several milliliters were removed from the total binding tube at 45 min (*arrow*) and added to another tube containing phentolamine (final concentration 10 μM). Duplicate 0.5-ml aliquots of this batch were then sampled at various times to measure reversal of [^{3}H]prazosin binding. In this figure, which is representative of three such experiments, 1000 cpm are equivalent to 94 fmoles of [^{3}H]prazosin bound per milligram of protein.

B. Analysis of kinetics of association of [^{3}H]prazosin with renal cortical membranes. Specific binding data from A are plotted as $\ln[B_{eq}/(B_{eq}-B_t)]$ versus time, where B_{eq} = counts per minute bound at equilibrium and B_t = counts per minute bound at time t. This analysis derives from the pseudo-first order nature of the conditions used in the experiment shown in A.

C. Analysis of kinetics of dissociation of specific binding of [^{3}H]prazosin. Dissociation data from A are plotted as $\ln[B_t/B_{eq}]$ versus time, where B_{eq} = counts per minute bound at equilbrium and B_t = counts per minute bound at time t.

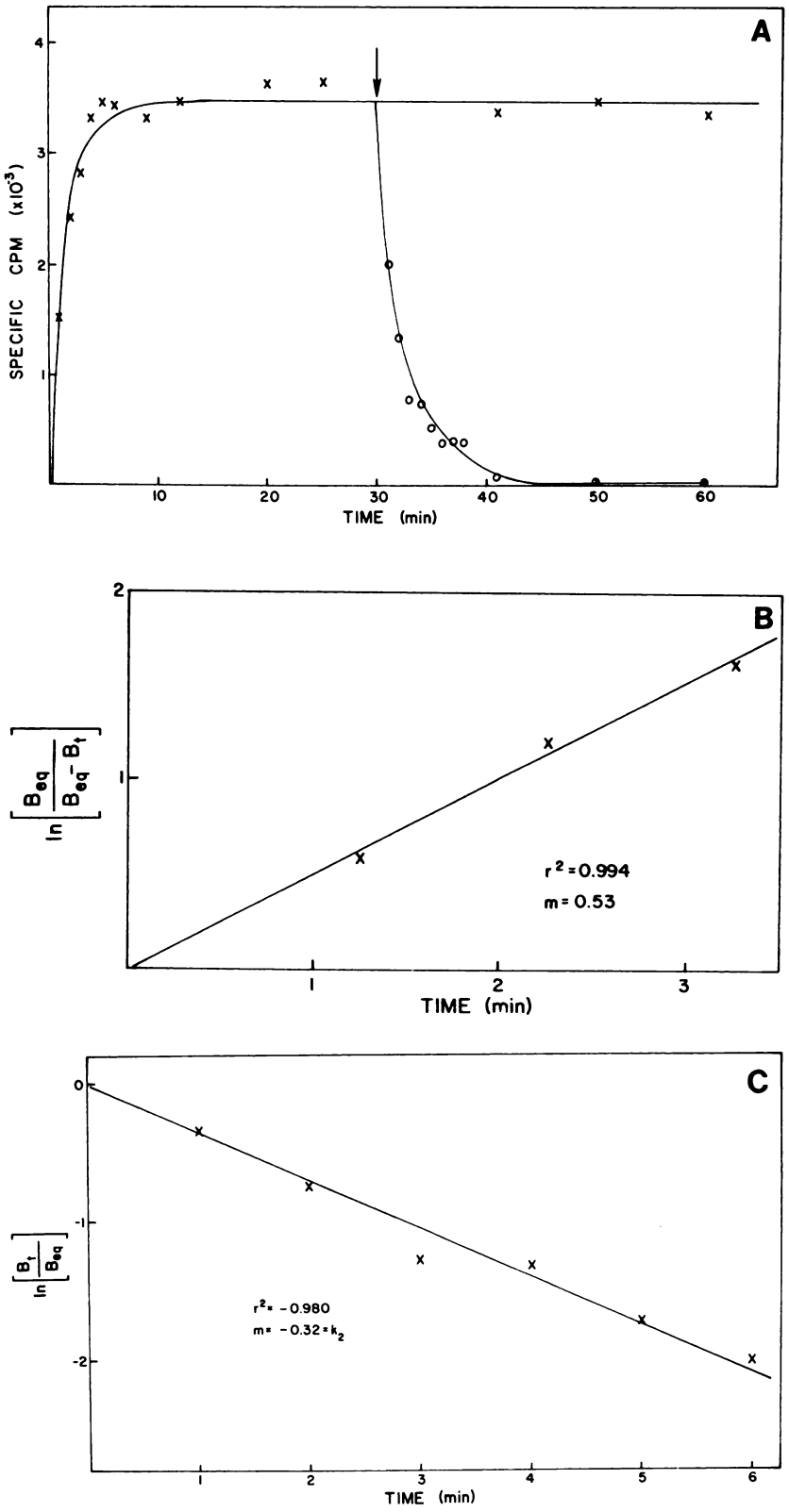

FIG. 2. *Kinetics of [³H]yohimbine binding to rat renal cortical membranes*

A. This experiment was performed exactly as that described in Fig. 1 except that [³H]yohimbine (11.8 nM) was used instead of [³H]prazosin, and 0.25-ml aliquots were removed from each batch. The dissociation phase of the experiment was started at 30 min (*arrow*). The data shown are representative of those obtained in two separate experiments; 3000 cpm are equivalent to 103 fmoles of [³H]yohimbine bound per milligram of protein.

B. Analysis of kinetics of association of [³H]yohimbine with rat renal cortical membranes, as described in Fig. 1B.

C. Analysis of kinetics of dissociation of [³H]yohimbine with rat renal cortical membranes, as described in Fig. 1C.

data, similar to those constructed for [^{3}H]prazosin, were constructed for [^{3}H]yohimbine binding (Fig. 2B and C). These plots yielded values for k_{obs} and k_2 of 0.53 min^{-1} and 0.32 min^{-1}, respectively. Values for k_1 and K_D were calculated as described above:

$$k_1 = \frac{k_{obs} - k_2}{[\text{ligand}]} = \frac{0.53\ min^{-1} - 0.32\ min^{-1}}{11.8 \times 10^{-9}\ M}$$

$$= 1.8 \times 10^{7}\ min^{-1}\ M^{-1}$$

$$K_D = \frac{k_2}{k_1} = \frac{0.32\ min^{-1}}{1.8 \times 10^{7}\ min^{-1}\ M^{-1}} = 1.8 \times 10^{-8}\ M$$

From the results of these kinetic experiments, a 60-min period was chosen as the incubation time for assays using [^{3}H]prazosin, and a 30-min period was chosen for assays using [^{3}H]yohimbine.

Adrenergic type and subtypes identified by [^{3}H]prazosin and [^{3}H]yohimbine. Competition for [^{3}H]prazosin and [^{3}H]yohimbine binding sites by enantiomers of epinephrine demonstrated the appropriate stereoselectivity for adrenergic receptors. (−)-Epinephrine was 30-fold more potent than its biologically inactive enantiomer, (+)-epinephrine, in competing for [^{3}H]prazosin sites and 10-fold more potent than (+)-epinephrine in competition for [^{3}H]yohimbine sites (Table 1).

The binding sites occupied by [^{3}H]prazosin and [^{3}H]yohimbine represented *alpha*-adrenergic receptors as shown by the appropriate rank order of potency of adrenergic agents: phentolamine > (−)-epinephrine ≥ (−)-norepinephrine > dopamine ≫ propranolol > isoproterenol (Table 1). Preincubation of membranes at 37° for 30 min to remove any endogenous ligands bound to the receptor did not enhance agonist affinities at either the *alpha*$_1$- or *alpha*$_2$-adrenergic receptors.

TABLE 1

Competition for [^{3}H]yohimbine and [^{3}H]prazosin binding to rat renal cortical membranes by adrenergic antagonists and agonists

Membranes were incubated with either [^{3}H]yohimbine or [^{3}H]prazosin and various concentrations of competing agents. Generally, 10 concentrations of each competing drug were used. The value for (−)-epinephrine was obtained in 10 studies with [^{3}H]yohimbine and 14 studies with [^{3}H]prazosin. Each competition curve was performed at least twice. K_D values for competing agents were calculated as described under Methods.

Agent	[^{3}H]Yohimbine		[^{3}H]Prazosin	
	K_D	Pseudo-Hill slope	K_D	Pseudo-Hill slope
	nM		*nM*	
Antagonists				
Phentolamine	25	1.08	15	0.90
Yohimbine	11	1.05	770	0.92
(−)-Propranolol	36,000	0.98	36,000	1.03
Prazosin	80	1.18	0	0.87
Indoramin	970	0.99	31	0.87
Agonists				
(−)-Epinephrine	630	0.56	341	0.63
(+)-Epinephrine	7,800	0.54	11,000	0.74
(−)-Norepinephrine	609	0.56	931	0.60
(−)-Isoproterenol	66,000	0.77	90,000	0.67
Dopamine	6,900	0.63	95,000	0.67
Clonidine	67	0.77	1,200	0.74
Phenylephrine	19,000	0.57	7,100	0.53
α-Methylnorepinephrine	110	0.68	6,358	0.68

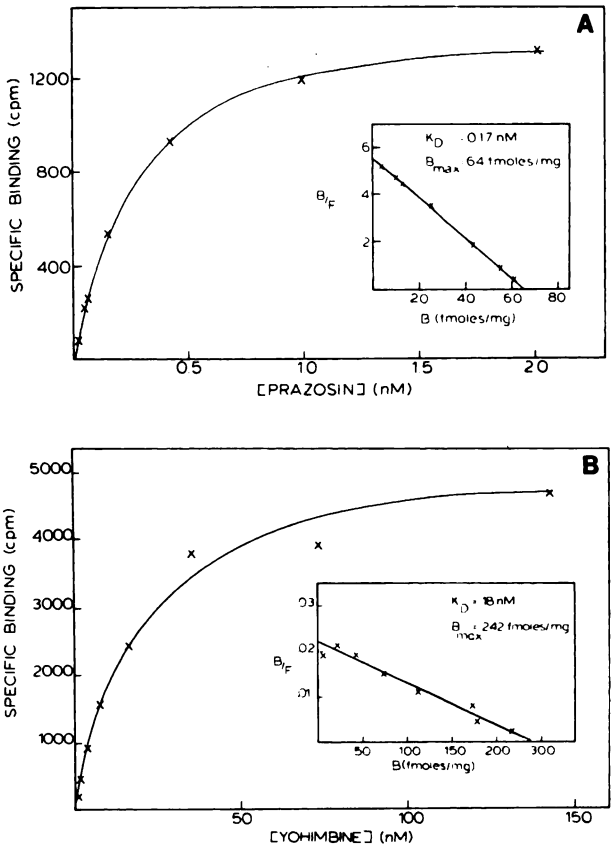

FIG. 3. *Binding isotherms and Scatchard analysis of [^{3}H]prazosin and [^{3}H]yohimbine binding to rat renal cortical membranes*

Membranes were incubated with increasing concentrations of radioligand in the absence (total binding) or presence (nonspecific binding) of 10 μM phentolamine. A, Saturation isotherm for binding of [^{3}H]prazosin; B, saturation isotherm for binding of [^{3}H]yohimbine. The *insets* show Scatchard analyses of binding isotherms. The data shown are representative of those obtained in seven experiments for [^{3}H]prazosin and nine experiments for [^{3}H]yohimbine.

Competition by agents selective for *alpha*-adrenergic subtypes demonstrated that [^{3}H]prazosin was binding to an *alpha*$_1$-adrenergic receptor (Table 1). Accordingly, prazosin and indoramin, *alpha*$_1$-selective antagonists, and phenylephrine, an *alpha*$_1$-selective agonist, were more effective at competing for [^{3}H]prazosin binding sites than were yohimbine, an *alpha*$_2$-selective antagonist, and clonidine and α-methylnorepinephrine, *alpha*$_2$-selective agonists. In contrast, competition curves with the same selective agents indicated that [^{3}H]yohimbine was binding to an *alpha*$_2$-adrenergic receptor (Table 1).

In general, at both *alpha*-receptor subtypes, the pseudo-Hill slopes for the agents competing for radioligand binding sites were close to unity for antagonists but were considerably lower (0.5–0.8) for agonists.

Saturation of binding isotherms with [^{3}H]prazosin and [^{3}H]yohimbine and effects of NaCl on radioligand binding. Both [^{3}H]prazosin binding and [^{3}H]yohimbine binding were saturable with ligand concentration, and Scatchard analysis of the binding isotherms revealed only one class of binding sites (Fig. 3A and B). In seven

TABLE 2

Affinity and Number of [^{3}H]prazosin and [^{3}H]yohimbine binding sites on rat renal cortical membranes: effect of Na^+ on binding

Membranes were incubated with varying concentrations of radioligand in the absence (total binding) and the presence (nonspecific binding) of 10 μM phentolamine. Scatchard analyses were then performed on the saturation isotherms thus generated to yield the K_D and B_{max} for binding. Values are means ± standard error of the mean. *n*, Number of separate experiments.

Treatment	*n*	K_D	B_{max}	Hill slope
		nM	*fmoles/mg*	
Buffer alone	4	0.39 ± 0.12	85 ± 11	1.05 ± 0.07
Prazosin buffer + 100 mM NaCl	3	0.32 ± 0.08	105 ± 24	1.03 ± 0.03
All experiments	7	0.36 ± 0.08	93 ± 12	1.04 ± 0.04
Buffer alone	6	25 ± 7.0	193 ± 14	1.03 ± 0.03
Yohimbine buffer + 100 mM NaCl	9	18 ± 3.0	270 ± 17[a]	1.00 ± 0.02
All experiments	15	20 ± 3.0	239 ± 15	1.02 ± 0.02

[a] $p < 0.01$ compared with buffer alone.

experiments, the mean K_D for binding of [^{3}H]prazosin to rat renal cortical membranes was 0.36 nM (± 0.08 SEM). The B_{max}, the maximal number of [^{3}H]prazosin binding sites, was 93 fmoles/mg of protein (± 12 SEM) (Table 2). In 15 experiments, the mean K_D and B_{max} for [^{3}H]yohimbine binding were 20 nM (± 3 SEM) and 239 fmoles/mg of protein (± 15 SEM), respectively (Table 2). However, as illustrated in Table 2, the presence or absence of sodium in the binding assay influenced the number of binding sites detected by [^{3}H]yohimbine. The addition of 100 mM NaCl increased the number of [^{3}H]yohimbine binding sites approximately 30%. The presence or absence of sodium had no significant effect on the K_D or B_{max} of [^{3}H]prazosin binding or on the K_D of [^{3}H]yohimbine. In addition, the K_D derived from equilibrium experiments agreed very well with that obtained from kinetic experiments for both radioligands (Figs. 1 and 2).

TABLE 3

Additivity of saturating concentrations of [^{3}H]prazosin and [^{3}H]yohimbine binding to rat renal cortical membranes

Membranes were added to saturating concentrations of each radioligand (4 nM [^{3}H]prazosin and 100 nM [^{3}H]yohimbine) in the presence or absence of 10 μM phentolamine. Specific binding was calculated for each ligand separately, and the sum of the specific binding of [^{3}H]prazosin and the specific binding of [^{3}H]yohimbine was compared with that calculated from the simultaneous addition of both radioligands at the above concentrations. Specific binding was also determined for membranes to which was added a saturating concentration of [^{3}H]prazosin (4 nM) in the presence of a saturating concentration (100 nM) of unlabeled yohimbine or a saturating concentration of [^{3}H]yohimbine (100 nM) in the presence of 4 nM unlabeled prazosin. The amount of radioligand specifically bound is shown as well as the ratio of [^{3}H]yohimbine sites to [^{3}H]prazosin sites ($alpha_2/alpha_1$). The data shown are representative of those obtained in five experiments performed in triplicate, which gave similar results.

Ligand	Total binding	Nonspecific binding	Specific binding	Amount specifically bound	$Alpha_2/alpha_1$
	cpm	*cpm*	*cpm*	*fmoles*	
[^{3}H]Prazosin	913	289	624	21.5	3.4
[^{3}H]Yohimbine	3229	2142	1087	72.5	
[^{3}H]Prazosin + [^{3}H]yohimbine (Predicted)	4144 (4142)	2474 (2431)	1670 (1711)	—	—
[^{3}H]Prazosin + unlabeled yohimbine (100 nM)	949	292	657	22.6	3.1
[^{3}H]Yohimbine + unlabeled prazosin (4 nM)	3474	2422	1052	70.1	

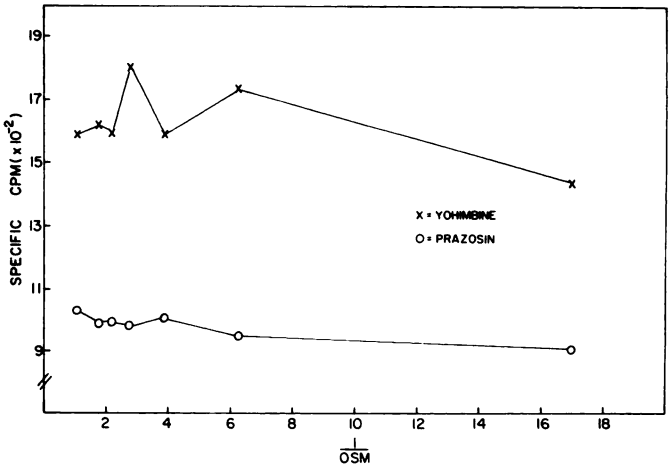

FIG. 4. *Effect of the tonicity of the incubation medium on the binding of [^{3}H]yohimbine and [^{3}H]prazosin to rat renal cortical membranes*

Membranes were incubated with 1.7 nM [^{3}H]prazosin or 13 nM [^{3}H]yohimbine in the absence (total binding) or presence (nonspecific binding) of 10 μM phentolamine. The tonicity of the incubation medium was altered by adding varying concentrations of sucrose in water (final concentrations 0–0.84 M). The data shown are from an experiment which was performed twice in triplicate; 1600 cpm of [^{3}H]yohimbine are equivalent to 108 fmoles bound per milligram of protein, and 1000 cpm of [^{3}H]prazosin are equivalent to 83 fmoles bound per milligram of protein.

In order to establish that [^{3}H]prazosin and [^{3}H]yohimbine were not binding to a common site, both radioligands were incubated with membranes separately at concentrations that would saturate their respective binding sites (5–10 times the K_D). The two radioligands were also added together in a separate set of tubes and incubated with membranes. The radioactivity specifically bound to the filters when the ligands were added together was the same as the sum of the radioactivity bound by each ligand separately (Table 3). Furthermore, incubation of each of the unlabeled selective antagonists at a concentration saturating its respective receptor subtype together with a saturating concentration of the other subtype-selective radiolabeled ligand (i.e., [^{3}H]yohimbine with unlabeled prazosin and vice versa) gave results identical with those obtained with each of the labeled ligands alone. The results of these two types of experiments (Table 3) indicate that the two radioligands were not binding to a common site under the conditions used in this assay.

Effect of altered tonicity on [^{3}H]prazosin and [^{3}H]yohimbine binding. In order to eliminate the possibility that the binding of either or both radioligands observed in this preparation resulted from uptake into vesicles, the following experiment was performed. Membranes were incubated with radioligand in the presence of varying

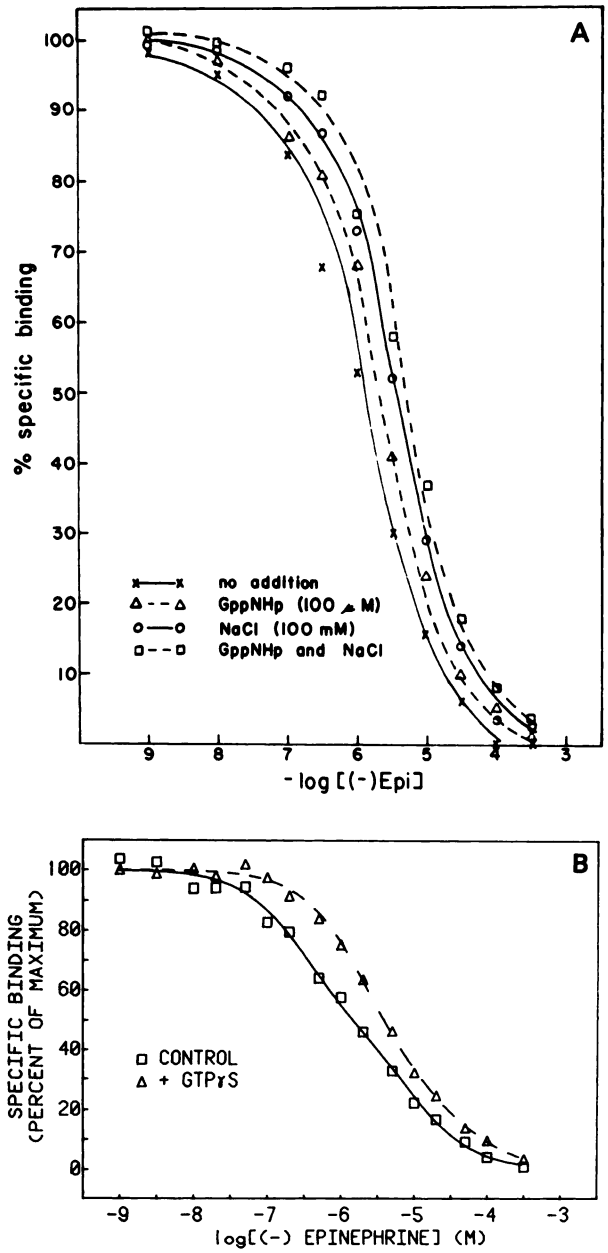

FIG. 5. *Effects of Gpp(NH)p, GTPγS, and NaCl on competition by epinephrine for [^{3}H]prazosin binding sites of rat renal cortical membranes*

Membranes were incubated with 1.0–2.0 nM [^{3}H]prazosin and varying concentrations of (−)-epinephrine in the presence or absence of the additions shown. Data in A represent the means of three experiments performed in triplicate; those in B, the means of a single experiment performed in triplicate in the absence and presence of 100 μM GTPγS.

concentrations of sucrose, and the effect of changing osmolarity on binding was determined. Since the amount of uptake into vesicles is dependent upon vesicle size, increasing the osmolarity of the binding medium would be expected to reduce uptake by causing osmotic shrinkage of the vesicles (18). Osmotic shrinkage would be expected to have little or no effect on binding, which is determined by receptor number per unit surface area and not by vesicle volume. As Fig. 4 shows, changing osmolarity (final sucrose concentrations 0–0.84 M) had virtually no effect on binding of either radioligand. Thus, in this membrane preparation, which showed no vesicular structures under the electron microscope prior to assay, vesicles were not re-forming during the assay under the conditions used.

Effects of guanyl nucleotides and sodium on competiton by epinephrine for [^{3}H]prazosin and [^{3}H]yohombine binding sites. The nonhydrolyzable GTP analogues Gpp(NH)p[1] and GTPγS (0.1 mM) caused small but reproducible decreases in the affinity of (−)-epinephrine for [^{3}H]prazosin binding sites (Fig. 5). In several experiments the shift in the competiton curve for (−)-epinephrine was about 2-fold and was accompanied by a slight but statistically significant increase in the pseudo-Hill slope (Table 4). Competiton of (−)-norepinephrine for [^{3}H]prazosin sites was also decreased 2-fold in the presence of 100 μM Gpp(NH)p. The addition of 100 mM NaCl shifted the agonist competition curve less than 2-fold, again with a statistically significant increase in the pseudo-Hill slope (Table 4). The simultaneous addition of 0.1 mM Gpp(NH)p and 100 mM NaCl decreased the affinity of (−)-epinephrine for the [^{3}H]prazosin binding site in an approximately additive manner (Fig. 5). The simultaneous addition of these agents had no effect on the ability of prazosin to compete for [^{3}H]prazosin binding (Table 4).

The addition of 100 μM Gpp(NH)p decreased the affinity of (−)-epinephrine 3-fold for [^{3}H]yohimbine binding sites and increased the pseudo-Hill slope (Table 4). GTPγS (100 μM) also decreased the affinity of (−)-epinephrine for [^{3}H]yohimbine sites (data not shown). GTP (1 mM) had a similar effect (Fig. 6B), although this effect was not consistently observed (Fig. 10). In the presence of the GTP-regenerating system used in the assay of adenylate cyclase, GTP consistently decreased the affinity of agonist for [^{3}H]yohimbine sites. The addition of 100 mM NaCl also decreased the affinity of (−)-epinephrine for [^{3}H]yohimbine binding sites of rat renal cortical membranes (Fig. 6). NaCl caused a parallel shift (i.e., similar pseudo-Hill slope) of about 2-fold in the agonist competition curve (Table 4) without altering the K_D of [^{3}H]yohimbine (Table 2). The simutaneous addition of Gpp(NH)p and NaCl decreased the affinity of (−)-epinephrine 10-fold, thus indicating a synergistic effect of these two agents (Fig 6). The pseudo-Hill slope obtained when Gpp(NH)p and NaCl were used together was not different from that obtained when Gpp(NH)p was used alone. The addition of Gpp(NH)p and NaCl had no effect on the affinity of yohimbine in competing for [^{3}H]yohimbine binding sites (Table 4).

Further studies indicated that the 2-fold decrease in agonist affinity for [^{3}H]prazosin sites produced by nucleotides was specific for guanyl nucleotides rather than adenyl nucleotides: App(NH)p (100 μM) and ATP (1 mM) did not alter competition by (−)-epinephrine for these sites, whereas Gpp(NH)p (100 μM), GTPγS (100 μM), and GTP (1 mM) did. As was the case for the [^{3}H]yohimbine binding sites, inclusion of a GTP-regenerating system was necessary to observe reproducibly this effect of GTP.

[1] The abbreviations used are: Gpp(NH)p, guanylyl imidodiphosphate; App(NH)p, adenylyl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

TABLE 4

Effects of NaCl and Gpp(NH)p on renal cortical alpha-adrenergic receptors

The indicated agents were incubated together with freshly prepared renal cortical membranes, [^{3}H]yohimbine, or [^{3}H]prazosin, and no addition, 100 mM NaCl, 100 μM Gpp(NH)p, or both 100 mM NaCl and 100 μM Gpp(NH)p. Generally, at least 10 concentrations of each competing drug were used and each concentration was assayed in triplicate. *n*, Number of times each experiment was performed. Values shown are means ± standard error of the mean.

Agent	*n*	[^{3}H]Yohimbine		[^{3}H]Prazosin	
		K_D	Pseudo-Hill slope	K_D	Pseudo-Hill slope
		nM		nM	
(−)-Epinephrine	9	820 ± 90	0.62 ± 0.04	410 ± 137	0.63 ± 0.02
(−)-Epinephrine + NaCl	6	1800 ± 100[a]	0.56 ± 0.02[b]	640 ± 115[a]	0.71 ± 0.03[a]
(−)-Epinephrine + Gpp(NH)p	9	2600 ± 400[a]	0.74 ± 0.03[a]	1000 ± 200[a]	0.71 ± 0.03[a]
Yohimbine	2	18	1.05	770	0.92
Yohimbine + NaCl + Gpp(NH)p	2	12	1.07	—	—
Prazosin	2	80	1.18	0.85	0.87
Prazosin + NaCl + Gpp(NH)p	2	—	—	1.14	0.88

[a] $p < 0.05$ compared with control by the paired *t*-test (one tailed).
[b] Not statistically significant compared with control.

The chloride salts of Na^+, Li^+, and K^+ (at 100 mM) were able to decrease the affinity of (−)-epinephrine at [^{3}H]prazosin sites (Table 5). Given the small magnitude of this effect and the variability between animals and experiments, it was not possible to determine a rank order of potency for these monovalent cations. However, the effect was not merely due to ionic strength or tonicity because the addition of 100 mM *N*-methyl-D-glucamine did not alter the affinity of (−)-epinephrine for [^{3}H] prazosin binding sites (Table 5). *N*-Methyl-D-glucamine was adjusted to the proper pH (7.5) with concentrated HCl, so that the decrease in affinity of epinephrine produced by NaCl, LiCl, and KCl is probably not attributable to the chloride anion but rather to the monovalent cations.

Further characterization of the decreases in affinity of epinephrine for [^{3}H]yohimbine binding sites produced by NaCl and Gpp(NH)p was much easier because of the synergistic effect of these two agents (Fig. 6A). A prominent decrease in the affinity of (−)-epinephrine was noted with either NaCl or sodium acetate, whereas LiCl produced a smaller decrease and KCl had almost no effect (Fig. 7). The half-maximal concentrations producing the decrease in affinity of epinephrine by NaCl, sodium acetate, and LiCl were approximately 30 mM. Therefore, the full expression of the decrease in affinity of (−)-epinephrine for [^{3}H]yohimbine binding sites produced by monovalent ions appears to be Na^+-selective. The lack of effect seen with KCl indicates that the decrease in agonist affinity produced by NaCl was not due to effects of altered tonicity or ionic strength.

Figure 8A illustrates that Na^+, and not Li^+ or K^+, increased the specific binding of [^{3}H]yohimbine. Both NaCl and sodium acetate increased [^{3}H]yohimbine binding, with a half-maximal effect noted at about 3 mM. The increase in [^{3}H]yohimbine binding was reversible (Fig. 8B). The addition of NaCl to washed membranes that had been preincubated with NaCl increased [^{3}H]yohimbine binding as much as did the addition of NaCl to membranes that had not been exposed to NaCl. The Na^+-mediated increase in [^{3}H]yohimbine binding represents an increase in B_{max} without a change in K_D (Table 2).

The shift in agonist affinity for [^{3}H]yohimbine sites produced by nucleotides was specific for the guanyl nucleotides Gpp(NH)p and GTP (Figs. 6B and 9), although the effect of GTP was variably present in the absence of a GTP-regenerating system. None of the nucleotides tested had any effect on the specific binding of [^{3}H] yohimbine (data not shown).

Computer analysis of competition by (−)-epinephrine for [^{3}H]prazosin and [^{3}H]yohimbine binding sites. In order to discriminate further differences in the effects of guanyl nucleotides and NaCl on renal cortical *alpha*$_1$- and *alpha*$_2$-adrenergic receptors, we performed a preliminary computer analysis of competitive binding curves of (−)-epinephrine at [^{3}H]prazosin and [^{3}H]yohimbine sites. As described in Table 6, data from two or three experiments were analyzed simultaneously with the LIGAND program of Munson and Rodbard (17). Under control conditions, competition by epinephrine for both [^{3}H]prazosin and [^{3}H]yohimbine sites was uniformly better fit by a two-site model than by a one-site model. About 60–75% of both the [^{3}H]prazosin and [^{3}H]yohimbine sites bound (−)-epinephrine with high affinity (K_D ~ 250 nM), whereas the remainder of the sites had a low affinity (K_D ~ 5 μM) for (−)-epinephrine. The addition of guanyl nucleotides, which decreased the over-all affinity of (−)-epinephrine at both [^{3}H]prazosin and [^{3}H]yohimbine sites (Figs. 5, 6; Table 4), appeared to alter these two receptor subtypes differently: at the *alpha*$_1$-receptors, the nucleotides decreased the affinity of (−)-epinephrine at both the high- and low-affinity classes of sites, whereas at the *alpha*$_2$-receptors, the nucleotides increased the proportion of the low-affinity class of binding sites. The addition of NaCl altered the epinephrine competition curves in a more similar manner at *alpha*$_1$- and *alpha*$_2$-receptors: NaCl appeared to increase the proportion of binding sites having low affinity for epinephrine. In addition, NaCl also seemed to decrease the affinity of the low-affinity class of sites which were identified by [^{3}H] yohimbine. The addition of both Gpp(NH)p and NaCl together with (−)-epinephrine yielded data for [^{3}H]yohimbine that were best fit to a single class of sites whose apparent K_D was 11 μM.

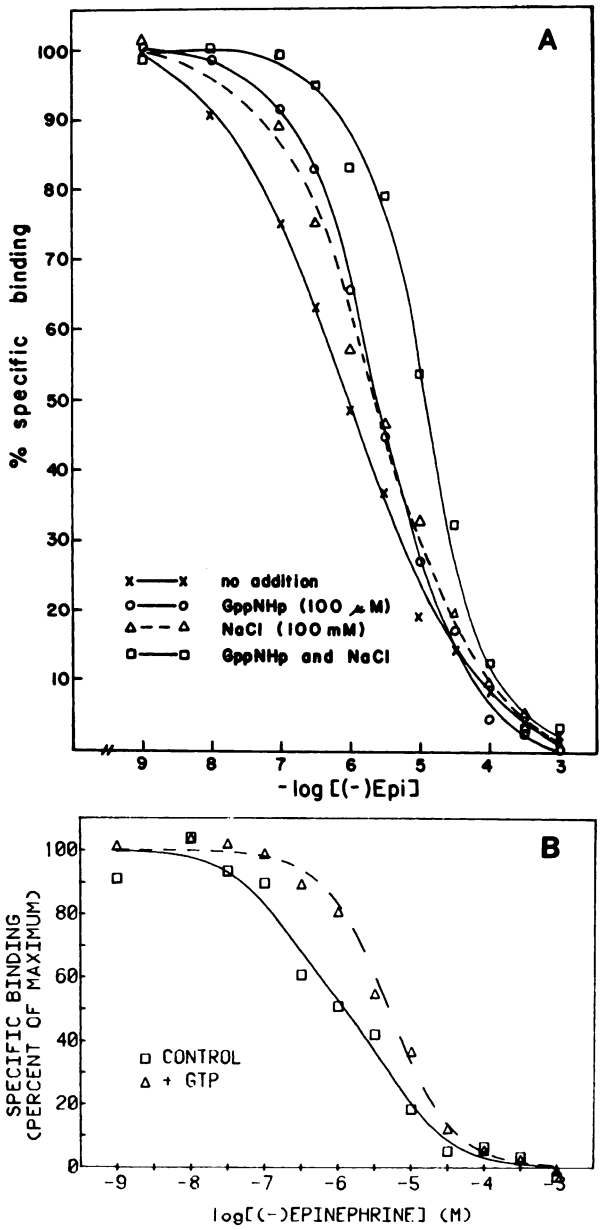

FIG. 6. *Effects of GTP, Gpp(NH)p, and NaCl on competition of epinephrine for [^{3}H]yohimbine binding sites of rat renal cortical membranes*

Membranes were incubated with 10–15 nM [^{3}H]yohimbine and varying concentrations of (−)-epinephrine in the presence or absence of the additions shown. Data in A represent the means of three experiments performed in triplicate; in B, the means of a single experiment performed in triplicate in the absence and presence of 1 mM GTP. The addition of GTP increased the pseudo-Hill slope from 0.62 to 0.82 in this experiment.

Lack of alpha-adrenergic-mediated inhibition of renal cortical adenylate cyclase. In an attempt to examine the functional role of *alpha*-adrenergic receptors in rat renal cortical membranes, we examined the effect of epinephrine on adenylate cyclase activity in response to a number of hormones (Fig. 10). As expected for renal cortical preparations (19), parathyroid hormone was the most efficacious activator of adenylate cyclase. Although *alpha*$_2$-adrenergic receptors inhibit adenylate cyclase activity in a number of other tissues (20–22), we observed

TABLE 5

Ionic effects on competition of epinephrine for [^{3}H]prazosin binding sites of renal cortical membranes

Membranes were incubated with [^{3}H]prazosin, varying concentrations of (−)-epinephrine, plus no addition, 100 mM NaCl, 100 mM KCl, 100 mM LiCl, or 100 mM *N*-methyl-D-glucamine. The latter compound was adjusted to pH 7.5 with concentrated HCl. K_D values shown are means ± standard error of the mean, calculated as described under Methods from three experiments. *N*-Methyl-D-glucamine was used in two experiments. By the paired *t*-test, $p < 0.02$ compared with the control value for both NaCl and KCl, and $p = 0.05$ for LiCl.

Agent	K_D
	nM
Epinephrine	898 ± 151
Epinephrine + NaCl	2089 ± 213
Epinephrine + KCl	2159 ± 146
Epinephrine + LiCl	3852 ± 1252
Epinephrine + *N*-methyl-D-glucamine	1038

no inhibition of basal enzyme activity or that stimulated by any of the six hormones examined (Fig. 10). The addition of 1 μM propranolol (to block the *beta*-adrenergic stimulation of adenylate cyclase activity produced by epinephrine; Fig. 10) or of up to 200 mM NaCl (in the presence of 100 μM GTP) failed to uncover an *alpha*-adrenergic inhibition of adenylate cyclase. The stimulation of the enzyme by either NaF or forskolin was also not inhibited by epinephrine plus propranolol (data not shown).

DISCUSSION

We have characterized the *alpha*$_1$- and *alpha*$_2$-adrenergic receptors in membranes from rat renal cortex using subtype-selective radioligands. Binding of [^{3}H]prazosin and [^{3}H]yohimbine show appropriate kinetics, saturability, and specificity, and appear to represent interaction of the ligands at receptors rather than uptake into vesicles. The rank order of potency of adrenergic agonists and antagonists demonstrates that [^{3}H]prazosin binds to

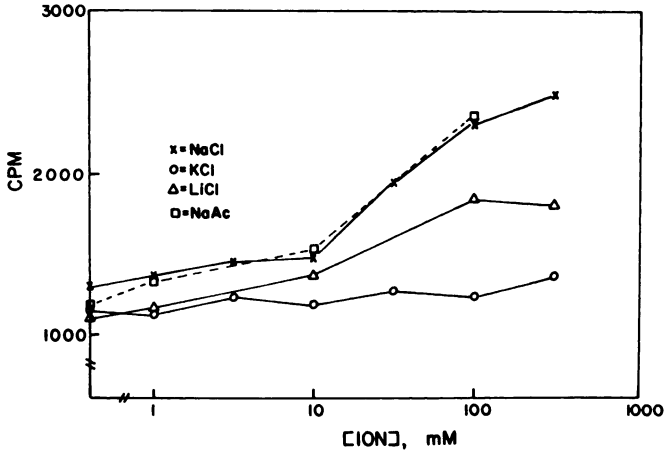

FIG. 7. *Effects of monovalent ions on agonist competition for [^{3}H] yohimbine binding to rat renal cortical membranes*

Membranes were incubated with 10–15 nM [^{3}H]yohimbine, 10^{-5} M (−)-epinephrine, and varying concentrations of ions. For the data shown, which are representative of those obtained on three occasions determined each time in triplicate, 2000 cpm are equivalent to 102 fmoles of [^{3}H]yohimbine bound per milligram of protein.

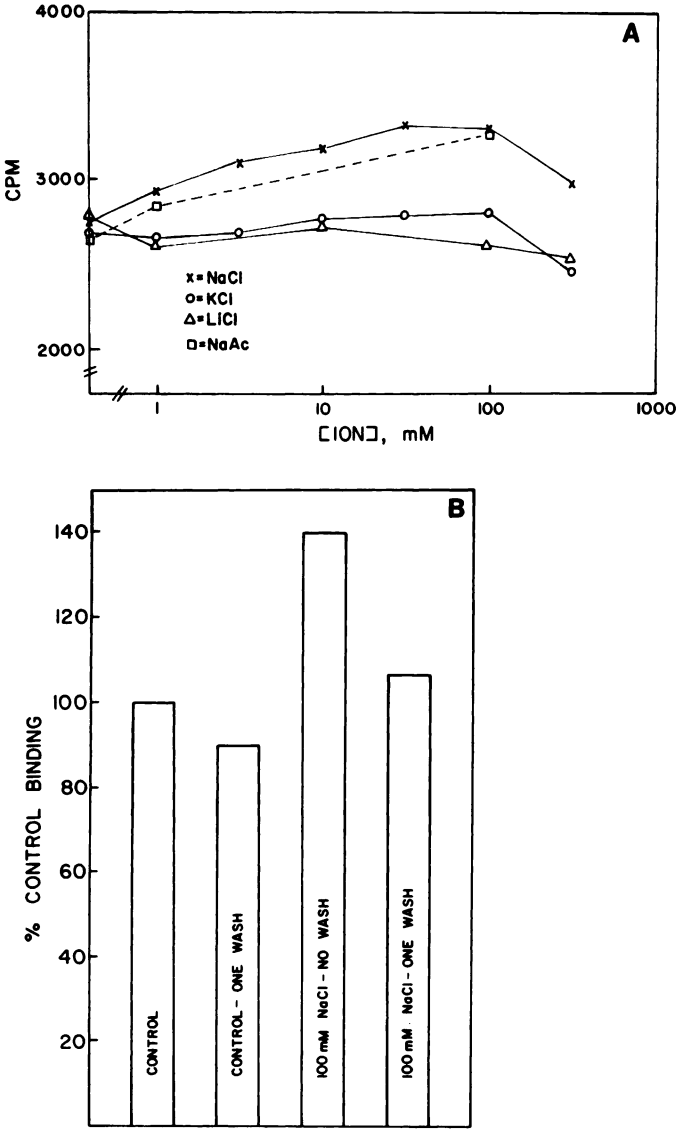

FIG. 8. *Effects of monovalent ions on specific binding of [^{3}H]yohimbine to rat renal cortical membranes*

A. Membranes were incubated with 10–15 nM [^{3}H]yohimbine in the presence or absence of 10 μM phentolamine. Varying concentrations of ions were added to both total and nonspecific binding tubes, and the difference between counts bound in the presence and absence of phentolamine was determined to investigate the effect of the ions on specific binding. For the data, which are representative of three such experiments performed in triplicate, 3000 cpm are equivalent to 152 fmoles of [^{3}H]yohimbine bound per milligram of protein.

B. Reversibility of the sodium effect on specific binding of [^{3}H] yohimbine. Control membranes were prepared as described under Methods and incubated with 13 nM [^{3}H]yohimbine in the presence or absence of 10 μM phentolamine and in the absence of NaCl. "Control—one wash" membranes were treated in a manner identical with that for control membranes except that they were washed once before incubation to determine loss of membrane during washing. NaCl membranes were treated in a manner identical with that for control membranes except that 100 mM NaCl was added prior to incubation. The "NaCl—one wash" membranes were exposed to 100 mM NaCl for 15 min; the NaCl was then removed by washing the membranes prior to incubation. Values are shown as percentages of binding to control membranes corrected for loss of milligrams of membrane protein due to washing. This experiment was performed in quadruplicate and was repeated twice with similar results.

TABLE 6

Effect of guanyl nucleotides and NaCl on competition by (−)-epinephrine for [^{3}H]prazosin and [^{3}H]yohimbine binding sites

Binding data in which (−)-epinephrine competed for [^{3}H]yohimbine and [^{3}H]prazosin sites in the absence or presence of either guanyl nucleotides and NaCl were analyzed with the iterative, nonlinear, curve-fitting program LIGAND (17). Data shown represent single values obtained from simultaneous analyses of two or three experiments (each involving 12–16 concentrations of (−)-epinephrine in triplicate). $\%R_H$ and $\%R_L$ refer to the percentage of high- and low-affinity sites resolved by the program, and K_{DH} and K_{DL}, the K_D values for the two classes of sites. All data were best fit by a model assuming the presence of two sites with differing affinities for (−)-epinephrine.

Ligand and addition	$\%R_H$	$\%R_L$	K_{DH}	K_{DL}
			nM	*nM*
[^{3}H]Prazosin				
None	66	34	222	5,069
100 μM GTPγS	77	23	858	9,373
300 μM Gpp(NH)p	79	21	818	13,248
[^{3}H]Prazosin				
None	75	25	312	7,755
100 mM NaCl	54	46	213	5,316
[^{3}H]Yohimbine				
None	61	39	190	4,392
100 μM Gpp(NH)p	30	70	114	3,762
100 mM NaCl	51	49	196	10,108

alpha$_1$-receptors whereas [^{3}H]yohimbine is selective for the *alpha*$_2$-subtype. Although prazosin is much more potent in competing for [^{3}H]yohimbine binding sites than is yohimbine in competing for [^{3}H]prazosin binding sites, binding of the two ligands is exactly additive, indicating that these two radioligands are not identifying a common binding site under the conditions used in this study. Both radioligands appear to bind to a single class of noncooperative sites. In addition, the two *alpha*-receptor subtypes are regulated differently by guanyl nucleotides and monovalent ions.

Although this is the first detailed characterization of *alpha*$_1$- and *alpha*$_2$-adrenergic receptors in the renal cortex obtained by using the radiolabeled antagonists [^{3}H]prazosin and [^{3}H]yohimbine, previous studies have reported radioligand binding to the two *alpha*-receptor subtypes in this tissue. Problems in these earlier reports include the use of labeled agonists such as [^{3}H]clonidine (3–5, 7) and [^{3}H]*p*-aminoclonidine (6), which may preferentially bind to a high-affinity subset of *alpha*$_2$-adrenergic receptors detectable in the absence of guanyl nucleotides (23–26), and identification of *alpha*$_1$-adrenergic receptors with [^{3}H]WB4101 (4), which may not be *alpha*$_1$-selective in peripheral tissues (27, 28). Similar problems do not seem to be evident for [^{3}H]yohimbine and [^{3}H]prazosin. With these radioligands, we found a 3-fold preponderance of *alpha*$_2$-receptors in renal cortical membranes. This contrasts strikingly with an earlier report of a 3-fold greater number of [^{3}H]WB4101 sites (*alpha*$_1$) than [^{3}H]clonidine sites (*alpha*$_2$) (4) in kidney membranes. Aside from the contribution of renal medullary membranes in that report, the discrepancy between our results and previous data is not difficult to explain: [^{3}H]WB4101 may be labeling both receptor sub-

types, giving an inflated value for the number of $alpha_1$-receptors, whereas [^{3}H]clonidine may not be labeling all of the $alpha_2$-receptors. Thus, our results underscore the importance of the use of highly selective antagonists to define accurately the number of adrenergic receptors of a particular receptor subtype.

While our manuscript was under review, a report appeared which demonstrated [^{3}H]prazosin and [^{3}H]yohimbine binding sites in rat renal cortical membranes (29) and a ratio between the number of sites for these two radioligands that was virtually identical with the value that we obtained. Schmitz *et al.* (29) also showed that the sum of [^{3}H]prazosin and [^{3}H]yohimbine sites was equal to the number of binding sites determined by using [^{3}H]dihydroergocryptine, a nonselective *alpha*-adrenergic antagonist. We have obtained similar results using this ligand.[2]

The preponderance of $alpha_2$-adrenergic receptors in the renal cortex may result from *alpha*-adrenergic receptors that have been suggested to regulate tubular reabsorption (1, 2), whereas $alpha_1$-adrenergic receptors may be those mediating gluconeogenesis (30) and renal vascular tone (1, 31). In recent autoradiographic experiments on $alpha_2$-adrenergic receptors in guinea pig kidney, Young and Kuhar (32) localized [^{3}H]clonidine binding predominantly to the proximal tubule. Because fluorescent histochemical studies have shown that adrenergic nerve endings are present in peritubular locations (33), the presence of $alpha_2$-adrenergic receptors on renal proximal tubules indicates that these receptors may be located in close proximity to adrenergic nerve endings. It is thus of interest that in additional studies we have found that 6-hydroxydopamine treatment, which destroys sympathetic neurons and reduces the renal norepinephrine content more than 85%, failed to alter either [^{3}H]yohimbine or [^{3}H]prazosin binding in renal cortical membranes.[2] This result suggests, but by no means proves, that the majority of renal *alpha*-adrenergic receptors are not located on presynaptic sympathetic nerves.

$Alpha_2$-adrenergic receptors are known to be coupled

[2] M. D. Snavely and P. A. Insel, unpublished observations.

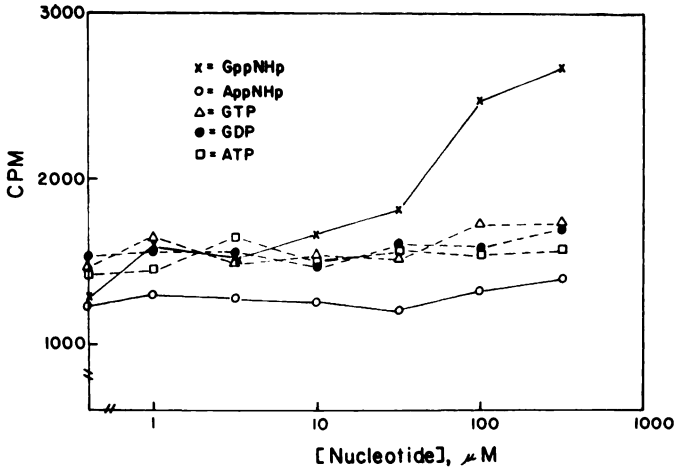

FIG. 9. *Effects of nucleotides on binding of epinephrine to [^{3}H] yohimbine sites of rat renal cortical membranes*

Membranes were incubated with [^{3}H]yohimbine (~10 nM), 10^{-5} M (−)-epinephrine, and varying concentrations of the nucleotides shown. In this experiment, 2000 cpm are equivalent to 85 fmoles of [^{3}H] yohimbine bound per milligram of protein.

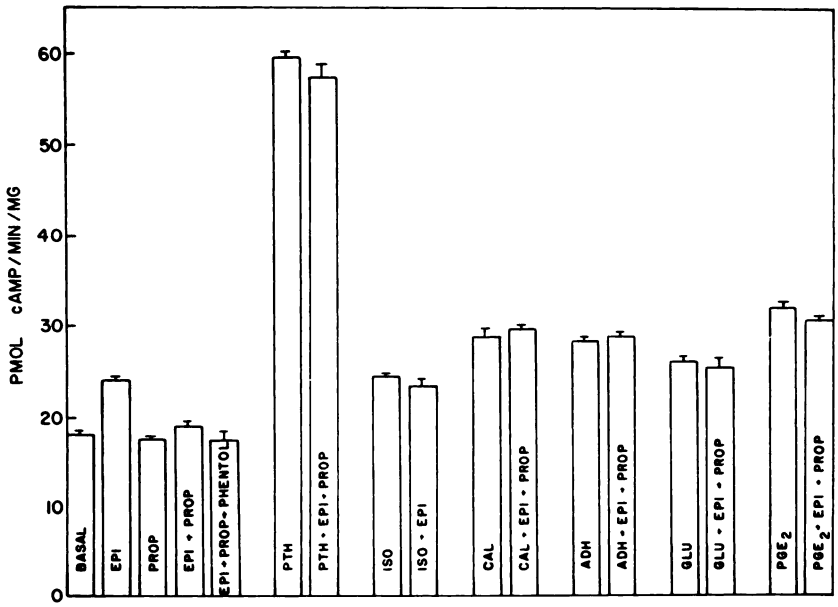

FIG. 10. *Effect of epinephrine on basal and hormone-stimulated adenylate cyclase activity*

Freshly isolated renal cortical membranes were incubated at 30° for 20 min with no addition, or in the presence of the following concentrations of hormones and other agents: *EPI*, (−)-epinephrine, 10 μM; *PROP*, (−)-propranolol, 1 μM; *PHENTOL*, phentolamine, 10 μM; *PTH*, parathyroid hormone, 10 μg/ml; *ISO*, (−)-isoproterenol, 10 μM; *CAL*, salmon calcitonin, 0.5 μg/ml; *ADH*, arginine vasopressin, 10 μM; *GLU*, glucagon, 17 μM; PGE_2, prostaglandin E_2, 100 μM. Error bars indicate the standard error of the mean of triplicate determinations. The data shown are representative of those obtained in five experiments.

to inhibition of adenylate cyclase in a variety of systems (e.g., refs. 20–22). Although the mechanisms which couple receptor occupancy to inhibition of adenylate cyclase are as yet ill-defined, it has been postulated that a nucleotide (GTP)-binding unit transduces the signal between the receptor and the catalytic enzyme (34). The evidence for such a mechanism comes from a variety of studies showing a guanyl nucleotide requirement for inhibition of adenylate cyclase as well as other studies showing guanyl nucleotide-mediated decreases in the affinity of agonists for "inhibitory" receptors (reviewed in ref. 34). We were thus not surprised to find that binding of epinephrine to [^{3}H]yohimbine binding sites in renal cortical membranes was decreased by the addition of GTP or the nonhydrolyzable GTP analogue Gpp(NH)p. Gpp(NH)p not only decreased the affinity of epinephrine at the *alpha*$_2$-receptor, but also increased somewhat the pseudo-Hill slope of the competition curve, indicating a change in the relative proportions of high- and low-affinity states of the receptor for agonist (8, 24). The fact that the affinity of epinephrine is inconsistently altered by GTP unless regenerating solution is included can be explained by the large amount of GTPase that may be variably present in our membrane preparations.

Because guanyl nucleotides decreased the affinity of epinephrine at [^{3}H]yohimbine binding sites, we attempted to localize these *alpha*$_2$-receptors by examining inhibition by epinephrine of adenylate cyclase activity stimulated by hormones that act at specific segments of individual nephrons (35). As shown in Fig. 10, we were able to stimulate adenylate cyclase activity with several hormonal agonists, but we were unable to produce any *alpha*$_2$-receptor-mediated inhibition of the enzyme. These results are in apparent disagreement with the results of Woodcock *et al.* (36), who observed *alpha*$_2$-mediated inhibition of basal adenylate cyclase activity in renal cortical membranes. The maximal inhibition of basal adenylate cyclase observed by Woodcock and coworkers was less than 20%, and the method of membrane preparation and the assay conditions used by Woodcock *et al.* (36) were quite different from those that we employed. Our basal enzyme activity was slightly higher than that obtained by Woodcock *et al.*, and our basal and hormone-stimulated activities are similar to those determined by other workers (37–39). Moreover, others have experienced difficulty in demonstrating *alpha*-adrenergic inhibition of renal adenylate cyclase activity (40).

From previous results, we expected to find no effect of guanyl nucleotides on competition by agonist for *alpha*$_1$-receptors recognized by [^{3}H]prazosin. *Alpha*$_1$-adrenergic receptors do not appear to be coupled to adenylate cyclase, and generally do not show an effect of nucleotides on agonist binding (8, 10, 13). Nevertheless we found a small, but consistent and highly reproducible, decrease in affinity of epinephrine for *alpha*$_1$-adrenergic receptors in the presence of Gpp(NH)p and GTP. We have, as yet, no explanation for this agonist-specific effect of guanyl nucleotides on renal *alpha*$_1$-adrenergic receptors. Conceivably, only a small fraction of the renal *alpha*$_1$-adrenergic receptors is regulated by guanyl nucleotides. Other workers have suggested that guanyl nucleotides may be regulating hepatic *alpha*-adrenergic receptors, the vast majority of which are of the *alpha*$_1$-subtype (11, 25); however, this effect of nucleotides on hepatic *alpha*$_1$-receptors is not generally accepted (10). Moreover, previous reports have indicated that guanyl nucleotides may be regulating other receptor types not thought to be "linked" to adenylate cyclase (41, 42). Taken together, the current observations and those of previous workers strongly suggest that guanyl nucleotide-binding proteins are likely to be involved in regulating binding (and presumably function) of agonists at a variety of receptor types but that the functional consequence of these agonists need not involve adenylate cyclase.

In addition to guanyl nucleotides, sodium also decreases the affinity of epinephrine in renal cortical membranes at both *alpha*$_1$- and *alpha*$_2$-receptors. The magnitude of the decrease was somewhat greater at *alpha*$_2$-receptors than at *alpha*$_1$-receptors, NaCl increased the pseudo-Hill slope more at *alpha*$_1$-receptors than at *alpha*$_2$-receptors, and the decrease was much more Na^+-selective at *alpha*$_2$-receptors than at *alpha*$_1$-receptors. The effects of sodium have previously been reported for *alpha*$_2$-adrenergic receptors (e.g., refs. 12, 13, 24), but we are aware of only a single preliminary report of a Na^+-mediated decrease in agonist affinity at *alpha*$_1$-adrenergic receptors (43). Although the mechanism for, and physiological significance of, the ionic effects on agonist binding to *alpha*$_1$- and *alpha*$_2$-receptors remain to be elucidated, it appears from the present study that Na^+, as well as guanyl nucleotides, can regulate agonist binding to the two *alpha*-receptor subtypes in kidney. Nevertheless, *alpha*$_2$-adrenergic receptors show much greater synergism between Na^+ and Gpp(NH)p than do *alpha*$_1$-adrenergic receptors. It is tempting to speculate that such synergism reflects different, albeit closely linked, sites of action for Na^+ and guanyl nucleotides acting on *alpha*$_2$-adrenergic receptors.

Preliminary computer modeling of (−)-epinephrine competition for [^{3}H]prazosin and [^{3}H]yohimbine binding sites indicates that although the effect of guanyl nucleotides is different at renal *alpha*$_1$- and *alpha*$_2$-adrenergic receptors, the effect of NaCl may be similar at the two subtypes. In support of previously published studies, guanyl nucleotides seem to shift high-affinity agonist sites to sites of low affinity (8, 23, 24, 44). This is reflected as a change in the relative percentages of high- and low-affinity sites without a change in K_D for (−)-epinephrine at either site. However, the effect of guanyl nucleotides at the *alpha*$_1$-adrenergic receptor appears to be a decrease in agonist affinity at both classes of sites with only a minor change in the percentages of these sites. The mechanism by which guanyl nucleotides alter agonist interaction at the *alpha*$_1$-adrenergic receptor, while clearly different from the effect at the *alpha*$_2$-receptor, remains to be elucidated.

The effect of NaCl on agonist binding, on the other hand, appears to be similar at the two renal *alpha*-receptor subtypes. The primary effect of the ion seems to be a redistribution of high- and low-affinity sites. This effect is somewhat different from that noted in studies of [^{3}H]dihydroergocryptine binding to platelet *alpha*$_2$-receptors (44), although NaCl may also alter the affinity of

(−)-epinephrine for the low-affinity site for agonist binding to the renal *alpha*$_2$-receptor. The reason for this difference and the mechanism by which Na^+ acts require further study.

The computer analysis that we present is described as preliminary because the power of resolution of this technique for analyzing binding data is in part dependent upon the purity of the system being studied. The crude homogenate that we used contains membranes from a variety of different cell types, and differences in the membrane environment of the receptors may enhance or obscure differences between the receptor entities themselves. Thus, in tissues such as renal cortex, models to which binding data can be fit may oversimplify complex and subtle differences among families of receptor subtypes. For this reason we present these results as tentative and believe that further computer analysis of the effects of guanyl nucleotides and NaCl on agonist binding to the *alpha*$_1$- and *alpha*$_2$-adrenergic receptors must await the preparation of membranes from purified cell types that contain one or both of the receptor subtypes.

Another difference between these two *alpha*-receptor subtypes in kidney is the selective effect of sodium in enhancing the B_{max} of [^{3}H]yohimbine binding but not that of [^{3}H]prazosin binding. This enhancement by Na^+ of antagonist binding to the *alpha*$_2$-receptor is readily reversible with washing, indicating that the ion must be present in the binding medium before enhanced *alpha*$_2$-receptor binding is apparent. The effect of sodium on antagonist binding at the *alpha*$_2$-receptor appears to be independent of the decrease in agonist affinity also caused by this ion for several reasons: (a) the change in antagonist binding, unlike the decrease in agonist affinity, is more ion-specific (cf. Fig. 7 and Fig. 8); (b) when (−)-epinephrine competition curves are normalized to 100% to correct for the increase in antagonist binding, a decrease in agonist affinity is still observed (Fig. 6); (c) the half-maximal concentrations for the two actions are different. The precise site of action of NaCl on *alpha*$_2$-adrenergic receptors will determine whether or not these two effects are important physiologically. If the ion is acting on the receptor extracellularly at the cell surface, the number of *alpha*$_2$-adrenergic receptors would probably be maximal all of the time and the receptors' affinity for epinephrine would be low. However, if the effects of NaCl are on the cytoplasmic surface of the plasma membrane, the regulation of *alpha*$_2$-receptors by Na^+ might vary under physiological and pathological conditions. Regulation of *alpha*-adrenergic receptors by Na^+ in the kidney may be of importance, particularly because the kidney maintains sodium balance and transports massive quantities of sodium across its membranes.

ACKNOWLEDGMENTS

We thank Lawrence C. Mahan for performing the electron microscopic examination of the renal cortical preparation, Arlene Koachman and Kay Snavely for technical assistance, and Sandy Dutky for typing the manuscript. We also thank Dr. Lee Limbird, who suggested the use of *N*-methyl-D-glucamine.

REFERENCES

1. Insel, P. A., and M. D. Snavely. Catecholamines and the kidney: receptors and renal function. *Annu. Rev. Physiol.* **43**:625–36 (1981).
2. Kim, J. K., S. L. Linas, and R. W. Schrier. Catecholamines and sodium transport in the kidney. *Pharmacol. Rev.* **31**:169–178 (1980).
3. Jarrett, B., W. J. Louis, and R. J. Summers. Characteristics of [^{3}H]clonidine binding to an α-adrenoceptor in membranes from guinea pig kidney. *Br. J. Pharmacol.* **65**:663–670 (1979).
4. U'Prichard, D. C., and S. H. Snyder. Distinct α-noradrenergic receptors differentiated by binding and physiological relationships. *Life Sci.* **24**:79–88 (1979).
5. Summers, R. J. [^{3}H]Clonidine binding to α-adrenoceptors in membranes prepared from regions of guinea pig kidney: alteration by monovalent and divalent cations. *Br. J. Pharmacol.* **71**:57–63 (1980).
6. Rouot, B. R., and S. H. Snyder. [^{3}H]Para-amino-clonidine: a novel ligand which binds with high affinity to α-adrenergic receptors. *Life Sci.* **25**:769–774 (1979).
7. McPhersen, G. A., and R. J. Summers. [^{3}H]Prazosin and [^{3}H]clonidine binding to α-adrenoceptors in membranes prepared from regions or fat kidney. *J. Pharm. Pharmacol.* **33**:189–191 (1981).
8. Hoffman, B. B., D. Mullikin-Kilpatrick, and R. J. Lefkowitz. Heterogeneity of radioligand binding to α-adrenergic receptors: analysis of guanine nucleotide regulation of agonist binding in relation to receptor subtypes. *J. Biol. Chem.* **255**:4645–4652 (1980).
9. Karliner, J. S., P. Barnes, C. A. Hamilton, and C. T. Dollery. α_1-Adrenergic receptors in guinea pig myocardium: identification by binding of a new radioligand, [^{3}H]prazosin. *Biochem. Biophys. Res. Commun.* **90**:142–149 (1979).
10. Hoffman, B. B., D. F. Dukes, and R. J. Lefkowitz. Alpha-adrenergic receptors in liver membranes: delineation with subtype-selective radioligands. *Life Sci.* **28**:265–272 (1981).
11. Geynet, P., N. Ferry, A. Borsodi, and J. Hanoune. Two distinct α_1-adrenergic receptor sites in rat liver: differential binding of (−)[^{3}H]norepinephrine, [^{3}H]prazosin and [^{3}H]dihydroergocryptine. *Biochem. Pharmacol.* **30**:1665–1675 (1981).
12. Motulsky, H. J., S. J. Shattil, and P. A. Insel. Characterization of α_2-adrenergic receptors on human platelets using [^{3}H]yohimbine. *Biochem. Biophys. Res. Commun.* **97**:1562–1570 (1980).
13. Tharp, M. D., B. B. Hoffman, and R. J. Lefkowitz. α-Adrenergic receptors in human adipocyte membranes: direct determination by [^{3}H]yohombine binding. *J. Clin. Endocrinol. Metab.* **52**:709–714 (1981).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
15. Salomon, Y. Adenylate cyclase assay. *Adv. Cyclic Nucleotide Res.* **10**:35–55 (1979).
16. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_I) and the concentration of an inhibitor that causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
17. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach to characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
18. Hopfer, U., K. Nelson, J. Perrotto, and K. J. Isselbacher. Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* **248**:25–32 (1973).
19. Chase, L. R., and G. D. Aurbach. Renal adenyl cyclase: anatomically separate sites of parathyroid hormone and vasopressin. *Science (Wash. D. C.)* **159**:545–547 (1968).
20. Sabol, S., and M. Nirenberg. Regulation of adenylate cyclase of neuroblastoma × glioma hybrid cells by α-adrenergic receptors. *J. Biol. Chem.* **254**:1913–1920 (1979).
21. Garcia-Sainz, J. A., B. B. Hoffman, S. Li, R. J. Lefkowitz, and J. N. Fain. Role of alpha$_1$ adrenoceptors in the turnover of phosphatidylinositol and of alpha$_2$-adrenoceptors in the regulation of cyclic AMP accumulation in hamster adipocytes. *Life Sci.* **27**:953–961 (1980).
22. Steer, M. L., and A. Wood. Regulation of human platelet adenylate cyclase by epinephrine, prostaglandin E_1, and guanine nucleotides. *J. Biol. Chem.* **254**:10791–10797 (1979).
23. Shattil, S. J., M. McDonough, J. Turnbull, and P. A. Insel. Characterization of *alpha*-adrenergic receptors in human platelets using [^{3}H]clonidine. *Mol. Pharmacol.* **19**:179–183 (1981).
24. Lynch, C. J., and M. L. Steer. Evidence for high and low affinity α_2-receptors: comparison of [^{3}H]norepinephrine and [^{3}H]phentolamine binding to human platelet membranes. *J. Biol. Chem.* **256**:3298–3303.
25. El-Refai, M. F., P. F. Blackmore, and J. H. Exton. Evidence for two α-adrenergic binding sites in liver plasma membranes. *J. Biol. Chem.* **254**:4375–4378 (1979).
26. Hoffman, B. B., T. Michel, D. Mullikin-Kilpatrick, R. J. Lefkowitz, M. E. M. Tolbert, H. Gilma, and J. N. Fain. Agonist vs. antagonist binding of α-adrenergic receptors. *Proc. Natl. Acad. Sci. U. S. A.* **77**:4569–4572 (1980).
27. Hoffman, B. B., and R. J. Lefkowitz. [^{3}H]WB4101—caution about its role as an alpha-adrenergic subtype-selective radioligand. *Biochem. Pharmacol.* **29**:1537–1541 (1980).
28. Massingham, R., M. L. Dubocovich, N. B. Sheppersen, and S. Z. Langer. In vivo selectivity of prazosin but not of WB4101 for postsynaptic alpha$_1$-adrenoceptors. *J. Pharmacol. Exp. Ther.* **217**:467–474 (1981).
29. Schmitz, J. M., R. M. Graham, A. Sagalowsky, and W. A. Pettinger. Renal alpha-1 and alpha-2 adrenergic receptors: biochemical and pharmacological correlations. *J. Pharmacol. Exp. Ther.* **219**:400–406 (1981).

30. Kessar, P., and E. D. Saggerson. Evidence that catecholamines stimulate renal gluconeogenesis through an α_1-type of adrenoceptor. *Biochem. J.* **190**:119–123 (1980).
31. Drew, G. M., and S. B. Whiting. Evidence for two distinct types of postsynaptic α-adrenoceptor in vascular smooth muscle *in vivo*. *Br. J. Pharmacol.* **67**:207–215 (1979).
32. Young, W. S., and M. J. Kuhar. α_2-Adrenergic receptors are associated with renal proximal tubules. *Eur. J. Pharmacol.* **67**:493–495 (1980).
33. Barajas, L. Innervation of the renal cortex. *Fed. Proc.* **37**:1192–1201 (1978).
34. Rodbell, M. Role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* (*Lond.*) **284**:17–22 (1980).
35. Morel, F. Sites of hormone action in the mammalian nephron. *Am. J. Physiol.* **240**:F159–F164 (1981).
36. Woodcock, E. A., C. I. Johnston, and C. A. Olssen. Alpha-adrenergic inhibition of renal cortical adenylate cyclase. *J. Cyclic Nucleotide Res.* **6**:261–269 (1980)
37. Liang, C. T., T. Takenawa, and B. Sacktor. Regulation of hormone (PTH and PGE_1)-stimulated adenylate cyclase by renal cytosolic factors. *Mol. Cell. Endocrinol.* **21**:221–231 (1981).
38. Herman, C. A., T. V. Zenser, and B. B. Davis. Effects of alamethicin on hormonal activation of renal adenylate cyclase. *Biochem. Pharmacol.* **29**:51–55 (1980).
39. Shatz, L. J., I. L. Schwartz, E. Kinne-Saffran, and R. Kinne. Distribution of parathyroid hormone-stimulated adenylate cyclase in plasma membranes of cells of the kidney cortex. *J. Membr. Biol.* **24**:131–144 (1975).
40. Morel, F., M. Imbart-Teboul, and D. Chabardes. Distribution of hormone-dependent adenylate cyclase in the nephron and its physiological significance. *Annu. Rev. Physiol.* **43**:569–581 (1981).
41. Glossmann, H., A. Baukal, and K. J. Catt. Angiotensin II receptors in bovine adrenal cortex. *J. Biol. Chem.* **249**:664–666 (1974).
42. Cantau, B., S. Keppens, H. DeWulf, and S. Jard. *J. Receptor Res.* **1**:137–168 (1980).
43. Glossmann, H., and R. Hornung. α-Adrenoceptors in rat brain: sodium changes the affinity of agonists for prazosin sites. *Eur. J. Pharmacol.* **61**:407–408 (1980).
44. Michel, T., B. B. Hoffman, and R. J. Lefkowitz. Differential regulation of the $alpha_2$-adrenergic receptor by Na^+ and guanine nucleotides. *Nature* (*Lond.*) **288**:709–711 (1980).

Send reprint requests to: Dr. Paul A. Insel, Division of Pharmacology, Department of Medicine, M-013 H, University of California, San Diego, La Jolla, Calif. 92093.