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Alpha$_2$-Adrenergic Receptors in Neuroblastoma × Glioma Hybrid Cells

Characterization with Agonist and Antagonist Radioligands and Relationship to Adenylate Cyclase

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SUMMARY

The neuroblastoma × glioma NG 108-15 (108CC15) hybrid cell line was used to examine neural *alpha*$_2$-adrenergic receptors and the coupled biochemical response of adenylate cyclase [EC 4.6.1.1; ATP pyrophosphate lyase (cyclizing)] inhibition. Binding isotherms of the agonist (−)-[^{3}H]epinephrine ([^{3}H]EPI) (0.1–20.0 nM concentration range) in both NG 108-15 and bovine cortex membranes were linear and [^{3}H]EPI affinity constants were similar in both tissues. [^{3}H]*p*-aminoclonidine (0.1–20.0 nM) ([^{3}H]PAC) showed a 2 to 4-fold lower affinity at a single site in NG 108-15 membranes compared with its previously reported one-site interaction in rat brain. At NG 108-15 sites, guanyl-5′-yl imidodiphosphate lowered ^{3}H-labeled agonist binding, whereas GTP had a biphasic effect. High-affinity [^{3}H]PAC binding and its regulation by GTP was enhanced by 1.0 mM Mg^{2+}. The *alpha*$_2$-antagonist [^{3}H]yohimbine ([^{3}H]YOH) also labeled a single site with high affinity. Binding studies confirmed previous suggestions that NG 108-15 cells possess *alpha*-receptors only of the *alpha*$_2$-subtype: thus at NG 108-15 [^{3}H]PAC binding sites, (−)-norepinephrine (NE) was 30 times more potent than (+)-NE; (−)-NE and (−)-EPI were 20 times more potent than (−)-isoproterenol; and YOH was 4 and 60 times more potent than (2,6-dimethoxyphenyloxyethyl)aminomethyl-1,4-benzodioxane (WB-4101) and prazosin, selective *alpha*$_1$-antagonists in brain tissue. In addition, there was no detectable specific binding of either [^{3}H]WB-4101 or [^{3}H]prazosin. Similar alpha$_2$-receptor specificity was observed for [^{3}H]EPI and [^{3}H]YOH sites. At [^{3}H]YOH sites, agonist but not antagonist competitors exhibited apparent negative cooperativity (n_H = 0.5–0.7). PAC was a partial agonist at NG 108-15 *alpha*$_2$-receptors; it lowered basal and prostaglandin E_1-stimulated adenylate cyclase activity, but the extent of inhibition was only 50% of that observed for the full agonists (−)-EPI and (−)-NE. PAC was also able to reverse the inhibition caused by (−)-EPI. The affinities of most antagonists were quite similar for inhibition of [^{3}H]EPI, [^{3}H]PAC, or [^{3}H]YOH binding, and for reversal of adenylate cyclase inhibition. However, catecholamines were 2–3 orders of magnitude more potent in inhibiting [^{3}H]PAC and [^{3}H]EPI binding than in inhibiting either adenylate cyclase or [^{3}H]YOH binding. These pharmacological studies indicate that all three ligands label the *alpha*$_2$-receptor in NG 108-15 cell membranes that is coupled to adenylate cyclase in a GTP-dependent, inhibitory manner. The disparity between catecholamine IC_{50} values at [^{3}H]EPI and [^{3}H]PAC sites as compared with [^{3}H]YOH sites suggests that NG 108-15 *alpha*$_2$-receptors occur in two or more affinity states. In support of this model, the number of [^{3}H]YOH-labeled sites is significantly greater than the number of high-affinity [^{3}H]PAC or [^{3}H]EPI sites. IC_{50} values for agonists at [^{3}H]YOH sites and the adenylate cyclase response were very similar. The similar nature of GTP regulation and Mg^{2+} interactions at NG 108-15 and brain *alpha*$_2$-receptor sites supports the hypothesis that brain *alpha*$_2$-receptors are also negatively coupled to adenylate cyclase.

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INTRODUCTION

Sabol and Nirenberg (1) have recently shown that, in membranes from the neuroblastoma × glioma hybrid cell line NG 108-15 (108CC15), catecholamines inhibit adenylate cyclase activity via an interaction at an *alpha*-adrenergic receptor. These investigators also speculated that NG 108-15 *alpha*-receptors are of the $alpha_2$-type. $Alpha_2$-Adrenergic receptors are distinguished pharmacologically from the classical postjunctional $alpha_1$-receptor by a much greater affinity of the antagonist yohimbine as compared with the $alpha_1$-selective antagonist prazosin, and by the selectively high affinity of imidazoline drugs such as clonidine.

The relationship between *alpha*-receptor activation and cellular response is not as clearly defined as is the case with the *beta*-adrenergic receptor, activation of which stimulates adenylate cyclase (EC 4.6.1.1) activity. However, $alpha_2$-receptors in several peripheral tissues such as human platelets (2), hamster and human adipocytes (3), and rat hepatocytes[2] are coupled to adenylate cyclase in a GTP-dependent, inhibitory manner. In brain and liver membranes, [^{3}H]catecholamines and [^{3}H]imidazolines label sites with $alpha_2$-receptor characteristics (4, 5), where agonist affinities are modulated by guanine nucleotides (6), in a manner analogous to modulation of *beta*-adrenergic and glucagon receptors, which are directly coupled to adenylate cyclase (7). It has been suggested that brain $alpha_2$-receptors may be coupled to adenylate cyclase, and that coupling may be inhibitory or "negative" in nature (6).

Hoffman and Lefkowitz (8) have recently proposed, on the basis of studies of human platelet *alpha*-receptors, that $alpha_2$-receptors, like *beta*-receptors, generally occur in distinct conformational states differing with respect to agonist affinities (high- and low-affinity receptors). Although previous studies have shown that the affinities of agonist competitors are very high and can be modulated by GTP at brain $alpha_2$-receptors labeled with agonist radioligands (6), it is not yet clear whether the $alpha_2$-receptor in neural tissues adheres to the two affinity-state model as proposed by Lefkowitz and colleagues. Another problem in the delineation of brain $alpha_2$-receptor function is that the intrinsic activity of imidazolines is not well established at this receptor. Although clonidine and PAC[3] are weak partial agonists at platelet $alpha_2$-receptors (9, 10), [^{3}H]imidazolines label the same high-affinity state of brain $alpha_2$-receptors as [^{3}H]catecholamines, presumptive full-agonist ligands (4, 11).

One approach to circumventing the difficulties associated with characterizing brain $alpha_2$-receptor function, such as the lack of a well-defined receptor-mediated adenylate cyclase response, involves examination of *alpha*-receptors in cultured neural cell lines, where this response is demonstrable. In the present study, we have characterized the *alpha*-adrenergic receptor of NG 108-15 cells by ligand binding techniques and correlated binding parameters with the coupled biochemical response of adenylate cyclase inhibition. NG 108-15 cell membranes were shown to contain solely *alpha*-receptors of the $alpha_2$-subtype, which are coupled to adenylate cyclase and which exist in different affinity states. The data also lend further support to the hypothesis that brain $alpha_2$-receptors are negatively coupled to adenylate cyclase.

MATERIALS AND METHODS

Growth of cells and preparation of homogenates. NG 108-15 cells (108CC15), obtained from Drs. B. Hamprecht (Wurzburg, Germany) and M. Nirenberg (*National Institutes of Health*), were grown as previously described (1). Briefly stated, the cells were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with newborn calf serum (10%), hypoxanthine (0.1 mM), aminopterin (1.0 μM), and thymidine (16.0 μM). After reaching confluence, the cells were harvested in the same medium by gentle agitation of the flasks and prepared for use in either binding or adenylate cyclase assays. In these experiments, cells of passage 20–40 were routinely used. For binding assays, cells were first counted using a hemocytometer, subsequently lysed by rapid freezing in acetione/CO_2, and stored at −80° for future use. Immediately prior to use, disrupted cells were resuspended and homogenized in 50 mM Tris-HCl buffer (pH 8.0 at 0°), using a Brinkman Polytron PT-10 (setting 5, 30 sec). Membranes were washed by centrifuging three times for 10 min at 50,000 × *g*, with intermediate resuspensions in the same buffer.

For adenylate cyclase assays, cells were washed twice by centrifugation for 10 min at 34,000 × *g* in an ice-cold buffer containing 5 mM Tris-HCl (pH 7.4), 0.32 mM sucrose, and 1 mM $MgCl_2$; they were subsequently disrupted by rapid freezing and stored as above at −80° for use within 3 weeks of preparation. Immediately prior to use, the disrupted cells were thawed and resuspended as above in 50 mM Tris-HCl buffer (pH 8.0 at 0°), at a concentration of 2–4 mg of protein per milliliter.

Protein was determined by the method of Lowry *et al.* (12), with bovine serum albumin as standard.

Binding assays. [^{3}H]PAC binding at 25° to NG 108-15 membranes was measured in 50 mM Tris-HCl buffer containing 1.0 mM $MgCl_2$, as previously described for brain preparations (11), except that incubation to equilibrium was carried out for 40 min and nonspecific binding was defined by parallel incubations containing 1.0 μM phentolamine. [^{3}H]EPI binding was determined in the above buffer, with the additional presence of 1.0 mM pyrocatechol and antioxidants as described previously (13), after preincubation of the membranes for 15 min at 25° with 10 μM pargyline to inhibit monoamine oxidase activity. Nonspecific binding was that observed in the presence of 1.0 μM oxymetazoline. [^{3}H]YOH was incubated with membranes in 50 mM Tris-HCl buffer for 60 min at 25°. Nonspecific binding was defined by parallel incubations with 0.1 mM (−)-NE. For all ligands a 1.0-ml assay volume containing 0.5–1.0 mg of protein was used,

[2] K. H. Jacobs, personal communication.

[3] The abbreviations used are: [^{3}H]PAC, [^{3}H]*p*-aminoclonidine; [^{3}H]EPI, (−)-[^{3}H]epinephrine; [^{3}H]YOH, [^{3}H]yohimbine; NE, norepinephrine; PGE_1, prostaglandin E_1; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; WB-4101, (2,6-dimethoxyphenyloxyethyl)aminomethyl-1,4-benzodioxane; n_H, Hill coefficient; α_2(H) and α_2(L), high- and low-affinity states of the $alpha_2$-receptor, respectively.

and optimal specific binding was achieved in Tris-HCl buffers of pH 8.3–8.4 at 25° for [^{3}H]PAC and [^{3}H]EPI, and at pH 7.2 at 25° for [^{3}H]YOH. All assays were routinely conducted under these conditions. After incubation, the contents of the assay tubes were immediately filtered under vacuum over Whatman GF/B filters (presoaked with 1.0 mM pyrocatechol for [^{3}H]EPI assays) and rinsed three times with 5 ml of ice-cold Tris-HCl buffer (pH 8.0 at 4°). Filters were subsequently counted by liquid scintillation spectrometry at 35–40% counter efficiency to determine bound radioactivity. In some experiments, [^{3}H]PAC binding was determined using tissue prepared as for adenylate cyclase assays. The characteristics of [^{3}H]PAC binding were not changed in this situation.

Adenylate cyclase assays. Adenylate cyclase activity was routinely assayed as described by Salomon (14), with some modifications. Briefly stated, 100-μl reaction mixtures contained 50 mM Tris-HCl (pH 7.7), 1.0 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM cyclic AMP, 1.0 mM isobutylmethylxanthine, 10 μM GTP, bovine serum albumin (0.1 mg/ml), 20 mM phosphocreatine, 5 units of creatine phosphokinase, 0.1 mM pargyline to inhibit catecholamine degradation, and 1–2 μCi of [α-^{32}P]ATP. Where indicated, 10 μM PGE_1 was added, and all reactions were begun by the addition of 50–150 μg of protein to the assay tubes. Incubations were carried out for 10 min at 37° for basal activity and for 5 min at 37° for PGE_1-stimulated activity.

Ligands. [^{3}H]EPI (40–80 Ci/mmole), [^{3}H]PAC (40–55 Ci/mmole), and [^{3}H]YOH (80–90 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). [^{3}H]EPI was stored under nitrogen at 0° and diluted immediately before use with 0.1% ascorbic acid. [^{3}H]PAC was stored at 0° in ethanol/0.1 N HCl (9:1) and diluted in water; [^{3}H]YOH was stored in ethanol and diluted with 10^{-6} N HCl. [α-^{32}P]ATP (23–35 Ci/mmole) for adenylate cyclase assays was also obtained from New England Nuclear Corporation.

Drugs and reagents. Drugs were donated by the following sources: catecholamine isomers, Sterling-Winthrop (New York, N. Y.); oxymetazoline, Schering Corporation (Kenilworth, N. J.); phentolamine, CIBA-Geigy Corporation (Summit, N. J.); clonidine, Boehringer Ingleheim Ltd. (Ridgefield, Conn.); dihydroergocryptine, Sandoz Pharmaceuticals (East Hanover, N. J.); YOH, Regis Chemical Company, Morton Grove, Ill.); WB-4101, Ward Blenkinsop Ltd. (Wembley, England); prazosin, Pfizer Inc. (New York, N. Y.). PAC was obtained from Dr. Bruno Rouot (University of Strasbourg). ATP (phosphorylated from adenosine) free of GTP contamination was obtained from Sigma Chemical Company (St. Louis, Mo.). Other reagents were obtained from the pharmaceutical company of origin or from commercial sources.

RESULTS

Alpha-Receptor radioligand binding to NG 108-15 cell membranes. The potent antagonist ligands [^{3}H]WB-4101 and [^{3}H]prazosin have been shown to label selectively *alpha*$_1$-receptors in brain membranes (15, 16). In preliminary experiments under brain assay conditions, we observed no specific binding of [^{3}H]WB-4101 or [^{3}H]prazosin, over a 0.1–2.0 nM concentration range, to membranes from NG 108-15 cells, indicating the absence of *alpha*$_1$-receptors. To determine the presence of *alpha*$_2$-receptors, we used three radioligands, the physiological agonist [^{3}H]EPI and the imidazoline [^{3}H]PAC, which have been shown to label *alpha*$_2$-receptors selectively in brain (11, 13), and the antagonist [^{3}H]YOH, which selectively labels *alpha*$_2$-receptors with high affinity in human platelets (17) and, in the presence of a sodium-potassium buffer, in brain tissue (18).

[^{3}H]PAC binding. Specific phentolamine-displaceable binding of [^{3}H]PAC was observed in NG 108-15 membranes at low concentrations of ligand. Kinetic studies (Fig. 1) showed that at 25° association and dissociation of [^{3}H]PAC binding were rapid and monophasic, with respective half-times of 12 and 20 min. The dissociation rate constant (k_{-1}) was 0.033 min^{-1}. The association rate constant (k_1) was calculated from the equation $k_1 = (k_{ob} - k_{-1})/[[^3H]PAC]$ (13) to be 9.4×10^6 M^{-1} min^{-1}, and from two experiments the K_D derived from kinetic rate constants (k_{-1}/k_1) was 3.5 nM.

Specific binding at equilibrium (40 min) was saturable to an apparently single order of sites, with data from a representative experiment shown in Fig. 2. [Representative data for all radioligand saturation experiments (Figs. 2, 4, and 6) were derived from experiments performed at the same time using the same batch of NG 108-15 membranes.] From seven experiments, the mean value (± standard error of the mean) for the K_D of [^{3}H]PAC was 1.8 ± 0.58 nM, in good agreement with the kinetically derived K_D value. With 0.5–0.7 nM [^{3}H]PAC present, specific binding (approximately 800 cpm) was 60–80% of total binding. The maximal number of [^{3}H]PAC binding sites (B_{max}) was 105.0 ± 42.5 fmoles/mg of protein (seven experiments), or approximately 8600 ± 2300 sites per cell.

The pharmacological specificity and affinity constants of competitors of [^{3}H]PAC binding to NG 108-15 membranes were very similar to those for [^{3}H]PAC, [^{3}H]clonidine, and [^{3}H]EPI binding to brain membranes (11, 13), and were typical of an *alpha*$_2$-receptor interaction (Table 1). In inhibiting [^{3}H]PAC binding, (−)-EPI and (−)-NE showed equally high affinity (IC_{50} 3–5 nM), whereas (−)-isoproterenol was about 20 times less potent. [^{3}H]PAC binding was stereoselective, since (+)-NE was 30 times less potent than (−)-NE. The imidazoline derivatives, PAC and oxymetazoline, were as potent as the catecholamines (IC_{50} 1–5 nM), whereas clonidine appeared to be about 10 times weaker, and the nonspecific *alpha*-antagonists dihydro-α-ergocryptine and phentolamine were also potent, with IC_{50} values of 4 and 22 nM, respectively. However, the antagonists WB-4101 and prazosin, which are *alpha*$_1$-selective in brain tissue (15), were about 5 and 60 times less potent at [^{3}H]PAC binding sites than the *alpha*$_2$-selective antagonist YOH. Competition curves for both agonist and antagonist inhibitors of [^{3}H]PAC binding were generally steep (n_H about 1.0), indicating that [^{3}H]PAC sites were homogeneous with respect to both drug classes.

(−)-[^{3}H]EPI binding. Previous studies of [^{3}H]EPI interactions with rat and bovine brain *alpha*$_2$-receptors utilized (±)-[^{3}H]EPI of relatively low specific activity (10–15 Ci/mmole). In these experiments, the radioligand

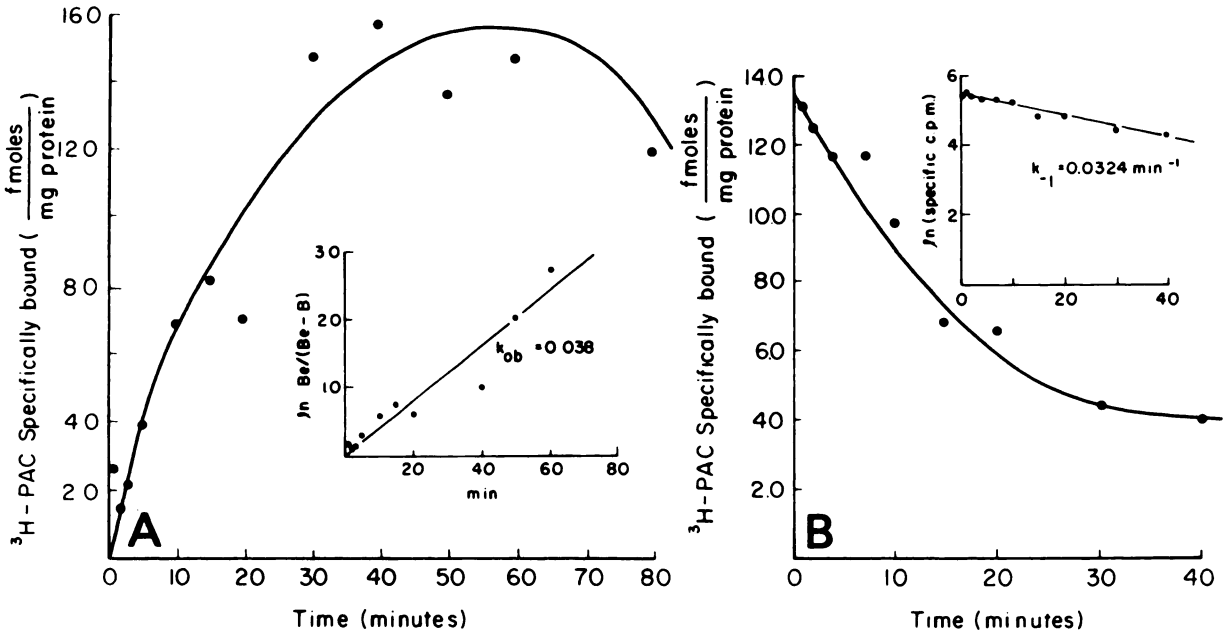

FIG. 1. *Time course of association and dissociation of [^{3}H]PAC specific binding to NG 108-15 membranes*

A. Association of [^{3}H]PAC specific binding (0.9 mg of protein) at 25° was measured at various intervals following addition of 0.5 nM [^{3}H]PAC. Specific binding at each point was defined as the difference between binding obtained in the absence and presence of 1.0 μM phentolamine. Points shown are for a single experiment performed in triplicate, which was replicated two times. *Inset*, pseudo-first-order kinetic plot of initial [^{3}H]PAC specific binding. The slope is equal to k_{ob}, the observed rate constant for the pseudo-first-order reaction.

B. Dissociation of specifically bound [^{3}H]PAC was measured at 25° following incubation with 0.5 nM [^{3}H]PAC (0.5 mg of protein) to equilibrium (40 min). At time zero, 1.0 μM phentolamine was added to the incubation mixtures and the reactions were terminated by filtration at various times. Nonspecific binding, determined in parallel samples containing 1.0 μM phentolamine, did not change during dissociation. Points shown are from a single experiment, performed in triplicate, which was replicated twice. *Inset*, semilogarithmic plot of dissociation.

appeared to label a single population of receptors (13). In the present study, we examined the binding of the active physiological isomer, (−)-[^{3}H]EPI (40–80 Ci/mmole) to NG 108-15 membranes. In kinetic experiments, specific (oxymetazoline-displaceable) binding of [^{3}H]EPI to NG 108-15 membranes was observed to associate somewhat more slowly at 25° than previously reported in bovine cortex, with $t_{1/2}$ of 7 min, reaching equilibrium by 60 min (Fig. 3A). As in preparations of bovine cortex, association at 0° was much slower (data not shown). After incubation to equilibrium with NG 108-15 membranes (60 min), [^{3}H]EPI specific binding was rather slowly dissociated at 25° by addition of 1.0 μM oxymetazoline, with $t_{1/2}$ of 14.5 min (cf. 3.5 min in bovine cortex). Unlike brain [^{3}H]EPI binding, dissociation of [^{3}H]EPI from NG 108-15 cells was monophasic, with a k_{-1} value of 0.047 min^{-1} (Fig. 3B). The association rate constant (k_1), derived as described above, was 2.4×10^7 M^{-1} min^{-1}, and the kinetically derived K_D (k_{-1}/k_1) was 3.9 nM.

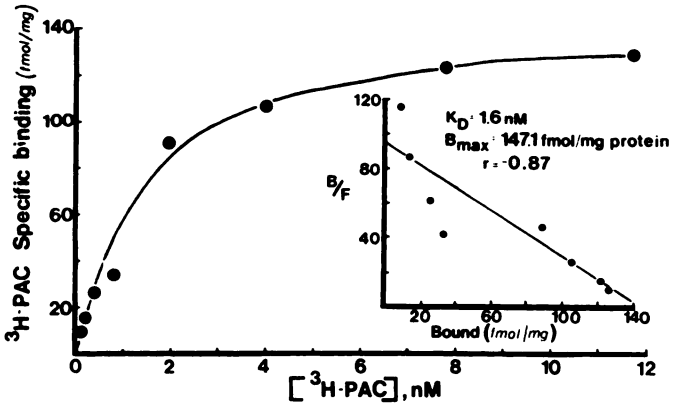

FIG. 2. *[^{3}H]PAC specific binding to NG 108-15 membranes as a function of increasing concentrations of [^{3}H]PAC*

Membranes (0.6 mg of protein) were incubated for 40 min at 25° with various concentrations of [^{3}H]PAC. Nonspecific binding was determined in the presence of 1.0 μM phentolamine, and increased linearly over the range of [^{3}H]PAC concentrations. Points shown are from a single experiment, performed in triplicate. *Inset*, Scatchard plot of saturation data.

[^{3}H]EPI specific binding to membranes from NG 108-15 cells at equilibrium was saturable, and a Scatchard (19) plot of the data appeared linear (Fig. 4), in agreement with previously observed single-site interactions of low specific activity (±)-[^{3}H]EPI in brain tissue (13). The K_D of [^{3}H]EPI from Scatchard analyses was 9.7 ± 5.4 nM (n = 7), somewhat greater than the kinetically derived K_D value. However, the values obtained approximated the K_D values for [^{3}H]EPI at brain *alpha*-receptor sites (13). The B_{max} of [^{3}H]EPI binding to NG 108-15 membranes was calculated to be 179.9 ± 59.8 fmoles/mg of protein (n = 7), or 14,600 ± 5,200 sites/cell, significantly higher than the corresponding value for [^{3}H]PAC binding. With 1.4 nM [^{3}H]EPI present, the concentration used in competition studies, specific binding (about 1,100 cpm) was 60–70% of total binding.

The pharmacological profile of [^{3}H]EPI binding to NG 108-15 membranes obtained from competition experiments was very similar to those of both NG 108-15 [^{3}H]PAC binding and to [^{3}H]EPI binding to brain *alpha*-receptor sites, with *alpha*$_2$-receptor specificity (YOH

TABLE 1

IC_{50} values for alpha$_2$-receptor agonists and antagonists in inhibiting [^{3}H]PAC, [^{3}H]EPI, and [^{3}H]YOH binding, and in inhibiting, or reversing the inhibition of basal and PGE_1-stimulated adenylate cyclase activity of NG 108-15 membranes

Drug inhibition of specific binding of 0.5 nM [^{3}H]PAC, 0.8 nM [^{3}H]YOH, and 1.4 nM [^{3}H]EPI, using six to eight concentrations of each unlabeled drug, was determined as described under Materials and Methods. IC_{50} values were determined graphically by log-probit analysis, and represent the means of two to four experiments, with binding at each drug concentration assayed in triplicate in each experiment. IC_{50} values for agonists in inhibiting basal or PGE_1-stimulated adenylate cyclase activity were determined graphically as above, under assay conditions described under Materials and Methods. IC_{50} values for antagonists represent their ability to reverse adenylate cyclase activity inhibition caused by 1.0 μM (−)-EPI. Values for effects on adenylate cyclase activity represent the means of two to six experiments. For any drug, IC_{50} values from individual experiments differed by less than 10% from each other.

Drug	IC_{50} for binding			IC_{50} for adenylate cyclase activity	
	[^{3}H]PAC	[^{3}H]EPI	[^{3}H]YOH	Basal	10 μM PGE_1
		nM			
Catecholamines					
(−)-NE	3.0	6.1	390	510	520
(−)-EPI	5.4	11.2	250	250	390
(−)-Phenylephrine	45	62	4,500	4,500	5,300
(−)-Isoproterenol	60	270	29,000	24,000	28,000
(+)-NE	90	32	1,900	380,000	100,000
Imidazolines					
PAC	1.3	4.0	52	660	430
Clonidine	30	32	48	470	415
Oxymetazoline	3.9	5.6	270	575	560
				Reversal	1 μM EPI
Antagonists					
Dihydro-α-ergocryptine	4.0	40	6.0	10	—
Phentolamine	22	65	39	10	—
YOH	36	140	7.8	40	—
WB-4101	120	350	16	—	—
Prazosin	2,100	5,500	42	—	—

more potent than prazosin), and low (nanomolar) IC_{50} values for catecholamines and imidazolines (Table 1). Again, as with NG 108-15 [^{3}H]PAC binding, steep competition curves indicated that both agonist and antagonist competitors recognized a homogeneous set of [^{3}H]EPI sites.

Ion and nucleotide interactions with [^{3}H]PAC and [^{3}H]EPI binding. In rat and bovine brain membranes, high-affinity binding of [^{3}H]EPI and [^{3}H]clonidine *alpha$_2$*-receptors is unaffected by Mg^{2+} added to the assay at 0.1–5 mM concentrations (6). However, the affinity of [^{3}H]PAC for human platelet *alpha$_2$*-receptor sites is en-

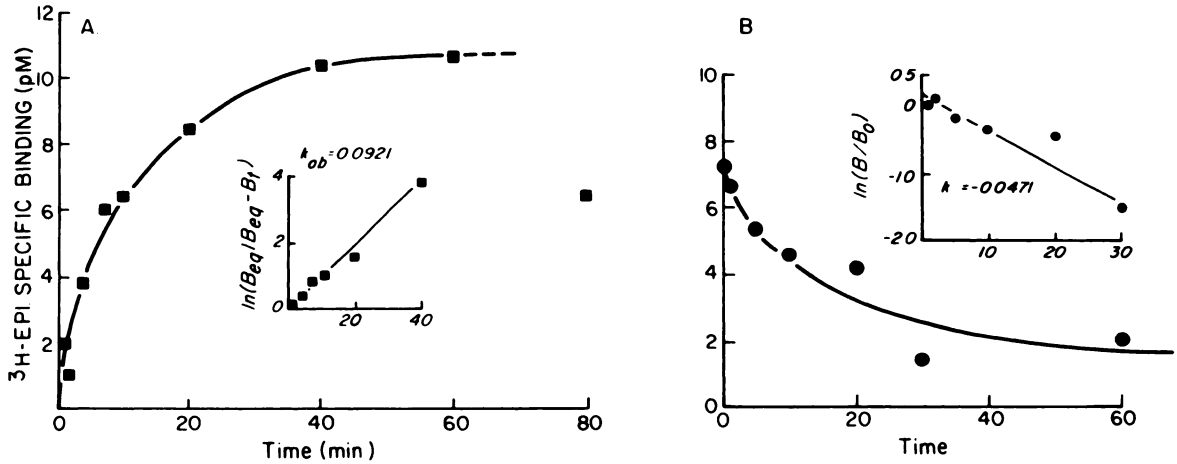

FIG. 3. *Time course of association and dissociation of [^{3}H]EPI specific binding to NG 108-15 membranes*

A. Association of [^{3}H]EPI specific binding to membranes (0.5 mg of protein) at 25° was measured at various intervals following the addition of 1.9 nM [^{3}H]EPI. Specific binding was defined as the difference between binding obtained in the absence and presence of 1.0 μM oxymetazoline. Points shown are for a single experiment, performed in triplicate, which was replicated twice. *Inset*, pseudo-first-order kinetic plot of initial [^{3}H]EPI specific binding.

B. Dissociation of specifically bound [^{3}H]EPI was measured at 25° following incubation with 1.9 nM [^{3}H]EPI to equilibrium (60 min). At time zero, 1.0 μM oxymetazoline was added to the incubation mixtures, and the reactions were terminated by filtration at various times. Nonspecific binding was determined in parallel samples containing 1.0 μM oxymetazoline. Points shown are from a single experiment, performed in triplicate, which was replicated twice. *Inset*, semilogarithmic plot of dissociation.

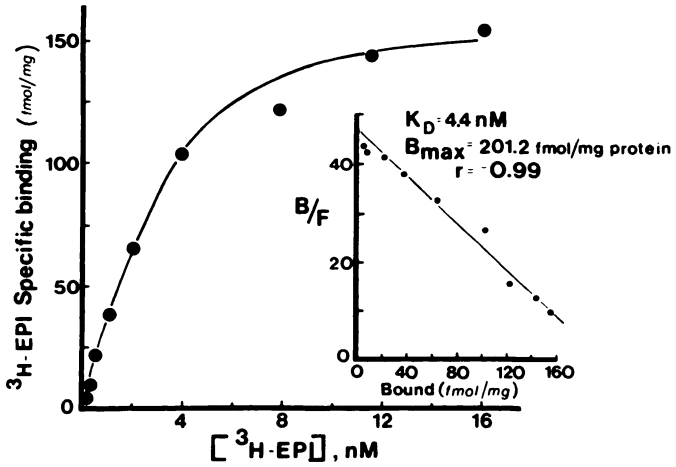

FIG. 4. *[^{3}H]EPI specific binding to NG 108-15 membranes as a function of increasing concentrations of [^{3}H]EPI*

Membranes (0.7 mg of protein) were incubated for 60 min at 25° with various concentrations of [^{3}H]EPI. Nonspecific binding was determined in the presence of 1.0 μM oxymetazoline, and increased linearly over the range of [^{3}H]EPI concentrations. Points shown are from a single experiment, performed in triplicate, using the same batch of membranes as in Fig. 2. *Inset*, Scatchard plot of saturation data.

hanced in the presence of increasing concentrations of Mg^{2+} (20). To determine whether [^{3}H]PAC specific binding to NG 108-15 membranes was Mg^{2+}-dependent we analyzed the saturation characteristics of [^{3}H]PAC binding in the absence of $MgCl_2$ and in the presence of 1.0 and 5.0 mM $MgCl_2$. The K_D for [^{3}H]PAC (no $MgCl_2$, 1.8 nM; 1.0 mM $MgCl_2$, 1.8 nM; 5.0 mM $MgCl_2$, 1.6 nM) was not affected. However, in this series of experiments, the presence of $MgCl_2$ increased the B_{max} of [^{3}H]PAC by approximately 20% (data not shown).

In the presence of 1.0 mM $MgCl_2$, GTP and GDP exhibited biphasic effects on [^{3}H]EPI and [^{3}H]PAC binding to NG 108-15 membranes (Fig. 5). ^{3}H-Labeled agonist specific binding was enhanced at lower concentrations of the nucleotides (0.1–10 μM range), whereas higher concentrations decreased ^{3}H-labeled agonist specific binding. Both effects appeared to result from changes in the number of high-affinity sites identified by ^{3}H-labeled agonists, while ^{3}H-labeled agonist affinities were unaltered. Thus, 10 μM GTP increased the B_{max} of [^{3}H]PAC by 17%, from 104 to 122 fmoles/mg of protein, with no change in K_D (1.1 versus 0.9 nM), whereas 50 μM GTP reduced the B_{max} by 27%, with again no change in K_D (1.1 nM with 50 μM GTP present). In contrast, the nonhydrolyzable analogue of GTP, Gpp(NH)p, inhibited [^{3}H]EPI and [^{3}H]PAC specific binding in a dose-dependent fashion over a wide concentration range, with ED_{50} values of 5.2 and 6.4 μM, respectively (Fig. 5). The biphasic interaction of GTP at agonist binding sites in the presence of Mg^{2+} is the same as that observed with [^{3}H]EPI and [^{3}H] clonidine specific binding to rat (6) and bovine brain membranes. In experiments performed in the absence of $MgCl_2$, 10 μM GTP still increased the B_{max} of [^{3}H]PAC binding to approximately the same extent, with no change in the K_D of [^{3}H]PAC (data not shown).

[^{3}H]YOH binding. The novel antagonist radioligand [^{3}H]YOH proved to be a good probe for *alpha*$_2$-receptors in NG 108-15 cells. Specific binding was 75–90% of total binding at concentrations of ligand lower than the K_D value. Association of specific binding at 25° was rapid, with equilibrium attained at after 10–20 min and maintained for up to 90 min of incubation; dissociation at 25° was rapid and complete within 60 min (data not shown). Saturation studies (Fig. 6) indicated that [^{3}H]YOH labeled a single class of sites with a mean K_D value of 9.1 ± 1.1 nM (n = 5) and a mean B_{max} of 258.4 ± 83.0 fmoles/

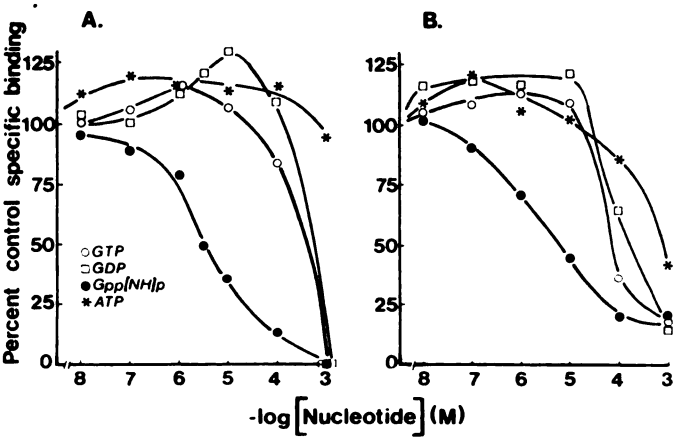

FIG. 5. *Effects of nucleotides on (A) [^{3}H]EPI and (B) [^{3}H]PAC specific binding to NG 108-15 membranes*

Membranes (0.5 mg of protein) were incubated with 1.4 nM [^{3}H]EPI or 0.5 nM [^{3}H]PAC for 60 min or 40 min, respectively, at 25° as described under Materials and Methods, in the absence or presence of various concentrations of nucleotides. Nonspecific binding was determined in the presence of 1.0 μM oxymetazoline and 1.0 μM phentolamine, respectively, and was not affected by different concentrations of nucleotides. Values are expressed as percentage of specific binding obtained in the absence of nucleotide, and represent the mean of four experiments.

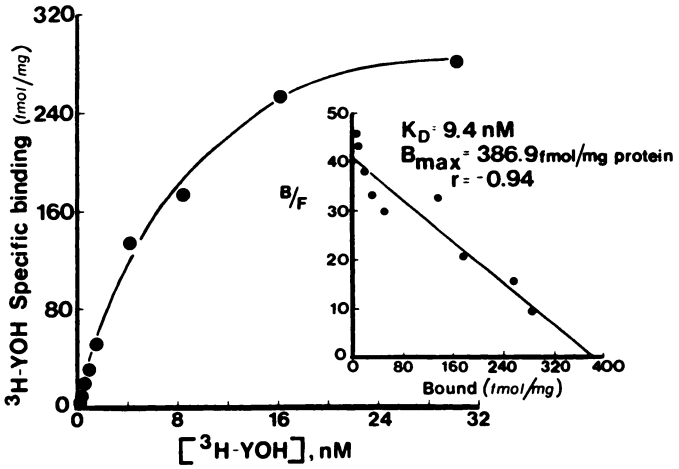

FIG. 6. *[^{3}H]YOH specific binding to NG 108-15 membranes as a function of increasing concentrations of [^{3}H]YOH*

NG 108-15 membranes (0.7 mg of protein) were incubated for 60 min at 25° with various concentrations of [^{3}H]YOH. Nonspecific binding was determined in the presence of 0.1 mM (−)-NE, and increased linearly over the range of [^{3}H]YOH concentrations. Points shown are from a single experiment, performed in triplicate, using the same batch of membranes as in Figs. 2 and 4. *Inset*, Scatchard plot of saturation data.

mg of protein, which represents 22,600 ± 5,000 sites/cell. Both the mean B_{max} data and the values obtained for the three ligands using the same preparation of cells (Figs. 2, 4, and 6) indicate that the rank order for ligand B_{max} values was [^{3}H]YOH > [^{3}H]EPI > [^{3}H]PAC.

In studies of the inhibition of [^{3}H]YOH binding by unlabeled competitors, specific [^{3}H]YOH binding appeared to have the characteristics of an *alpha*$_2$-receptor (Table 1). The antagonists YOH and phentolamine had potencies as competitors of [^{3}H]YOH binding similar to those for [^{3}H]PAC binding, and (−)-EPI, (−)-NE, clonidine, and PAC also showed high affinity for the site. In general, the IC_{50} values of these agents were very similar to values obtained in a more detailed study of [^{3}H]YOH binding to human platelet *alpha*$_2$-receptors (17). Although YOH was more potent than WB-4101 or prazosin in inhibiting NG 108-15 [^{3}H]YOH binding, suggesting an *alpha*$_2$-receptor interaction, it was noticeable that these three antagonists were significantly more potent inhibitors of [^{3}H]YOH binding than of [^{3}H]EPI or [^{3}H]PAC binding (Table 1). Conversely, as at platelet *alpha*$_2$-receptors, catecholamine agonists proved to be 50–100 times less potent as inhibitors of [^{3}H]YOH binding to NG 108-15 membranes than of [^{3}H]EPI or [^{3}H]PAC binding to the same membranes. The imidazolines PAC and oxymetazoline were similarly about 50 times less potent in inhibiting NG 108-15 [^{3}H]YOH sites. However, clonidine had approximately the same affinity at all three radioligand binding sites (Table 1).

In contrast to the steep competition curves of both unlabeled agonists and antagonists at [^{3}H]PAC and [^{3}H]EPI sites on NG 108-15 membranes, catecholamines and imidazolines competed at NG 108-15 [^{3}H]YOH sites with apparent negative cooperativity (Fig. 7A); i.e., they yielded shallow curves with pseudo-Hill coefficients of 0.5–0.7 (Fig. 7B). Similar heterogeneity of agonist and partial agonist interactions has been observed at human platelet [^{3}H]YOH sites (17). Antagonists such as yohimbine and dihydro-α-ergocryptine (Fig. 7), and WB-4101 and prazosin (not shown) exhibited, on the other hand, steeper competition curves at NG 108-15 [^{3}H]YOH sites with n_H values approximating 1.0.

To support the contention that [^{3}H]EPI, [^{3}H]PAC, and [^{3}H]YOH interact with the same population of NG 108-15 *alpha*$_2$-receptors, we performed experiments to determine whether guanine nucleotides would increase or decrease the potency of agonist competitors at [^{3}H]YOH sites in a manner analogous to the direct effects of the nucleotides on agonist ligand binding. GTP and Gpp(NH)p at concentrations as high as 100 μM had no effect on [^{3}H]YOH specific binding. 50 μM GTP or Gpp(NH)p, which inhibits ^{3}H-labeled agonist binding, increased the IC_{50} of both (−)-EPI and PAC at [^{3}H]YOH sites 2- to 3-fold. With 1.0 mM $MgCl_2$ present, 10 μM GTP, which *increased* [^{3}H]PAC binding (see above), reduced the IC_{50} values of (−)-EPI from 260 nM to 90 nM, and those of PAC from 50 nM to 20 nM, at [^{3}H]YOH sites. Thus nucleotide-induced increases and decreases in the number of high-affinity sites labeled by [^{3}H]PAC and [^{3}H]EPI are paralleled by increases and decreases in the affinity of PAC and (−)-EPI at [^{3}H]YOH sites.

Adenylate cyclase activity in NG 108-15 cells. Adenylate cyclase activity in NG 108-15 membranes was comparable to previously reported values in these cells (1), with a mean basal activity of 14.2 ± 0.8 pmoles· mg^{-1} min^{-1} (n = 15). The addition of 10 μM PGE_1 to NG 108-15 membranes resulted in a mean 9.2-fold elevation in activity to a value of 130.6 ± 13.6 pmoles· mg^{-1}min^{-1} (n = 9). Adenylate cyclase activity in NG 108-15 membranes was linear between 10 and 150 μg of protein, and for up to 15 min at 37° (data not shown).

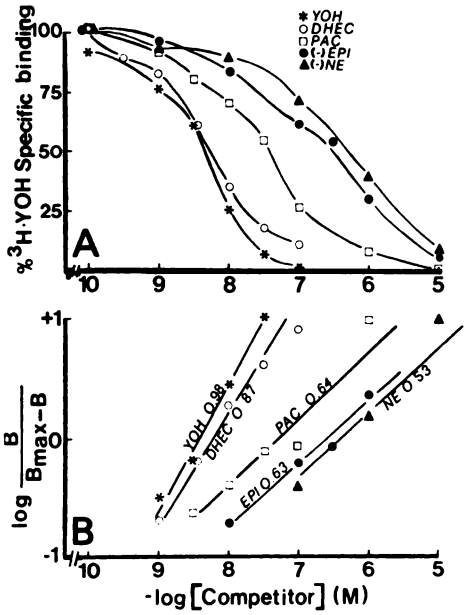

FIG. 7. *Inhibition of specific [^{3}H]YOH binding to NG 108-15 membranes by alpha-receptor agonists and antagonists*

A. Membranes (0.5 mg of protein) were incubated with 0.8 nM [^{3}H]YOH for 60 min at 25° in the presence of various concentrations of unlabeled drugs, and specific [^{3}H]YOH binding was determined as described under Materials and Methods. Values are expressed as percentage of specific binding observed in the absence of inhibitors, and are mean values from two to four experiments, each performed in triplicate.

B. Pseudo-Hill plots of competition data, with pseudo-Hill values (n_H) for each competitor. *DHEC*, dihydro-α-ergocryptine.

Both (−)-EPI and (−)-NE reduced basal and PGE_1-stimulated adenylate cyclase activity in NG 108-15 membranes (Fig. 8) by 40–50% in a dose-dependent manner, confirming previous reports (1). The inhibition of adenylate cyclase activity in NG 108-15 membranes by catecholamines appeared to be mediated via an *alpha*$_2$-receptor, since *alpha*$_2$-antagonist drugs could potently reverse the (−)-EPI-induced inhibition of basal adenylate cyclase activity (Table 1).

PAC appeared to act as a partial agonist at *alpha*$_2$-receptors in NG 108-15 cells (Fig. 9), as had previously been shown to be the case for clonidine (1). Over a 1.0 nM–100 μM concentration range, PAC lowered basal and PGE_1-stimulated adenylate cyclase activities with apparent IC_{50} values of approximately 0.7 and 0.4 μM, respectively. However, the extent of inhibition (25%) was only about one-half of that produced by the full agonists, (−)-EPI and (−)-NE over the same concentration range (Fig. 8). PAC was able, in a dose-dependent fashion, to reverse the inhibition caused by 1.0 μM (−)-EPI (Fig. 10),

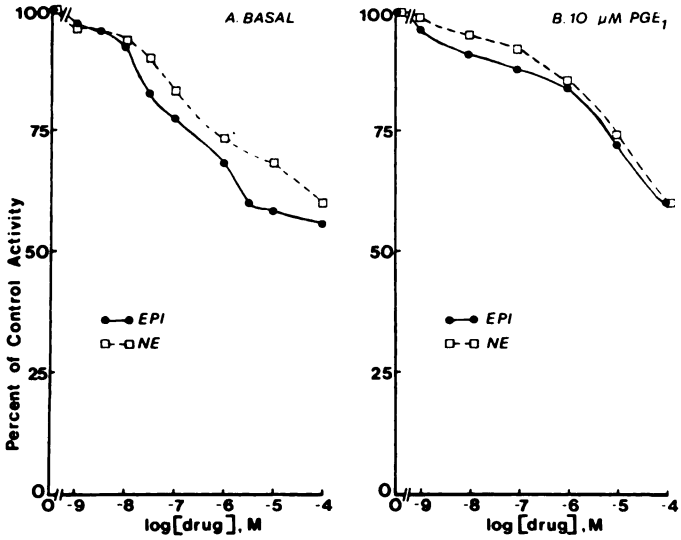

FIG. 8. *Inhibition of (A) basal and (B) PGE_1-stimulated adenylate cyclase activity by EPI and NE*

Adenylate cyclase activity at 37° was determined, as described under Materials and Methods, in the presence of various concentrations of drugs. Values are means from four to six experiments performed in duplicate, and are expressed as percentage of control activity measured in the absence of catecholamines. Mean control basal activity was 17.0 pmoles of cyclic [^{32}P]AMP/mg/min, and 10 μM PGE_1 produced a mean control activity of 107.6 pmoles of cyclic [^{32}P]AMP/mg/min.

further indicating that PAC acts as a mixed agonist-antagonist at NG 108-15 *alpha*$_2$-receptors. Inhibition of adenylate cyclase activity by catecholamines and imidazolines occurred over more than 2 orders of magnitude (Figs. 8 and 9), suggesting complex interactions.

The IC_{50} values for *alpha*$_2$-agonists in inhibiting adenylate cyclase (Table 1) and for *alpha*$_2$-antagonists in reversing (−)-EPI inhibition were similar to those reported previously (1). Although antagonist IC_{50} values were quite similar in the adenylate cyclase response and at the three radioligand binding sites in NG 108-15 membranes (Table 1), catecholamine IC_{50} values corresponded reasonably well only between the cyclase response and the [^{3}H]YOH site, whereas these agents were 2–4 orders of magnitude more potent at [^{3}H]PAC and [^{3}H]EPI sites.

DISCUSSION

The present experiments demonstrate that the agonist and antagonist radioligands [^{3}H]EPI, [^{3}H]PAC, and [^{3}H]YOH label *alpha*-adrenergic receptors on NG 108-15 cell membranes. The pharmacological properties of these binding sites (Table 1) and the absence of [^{3}H]prazosin or [^{3}H]WB-4101 binding indicate that NG 108-15 cells contain only receptors of the *alpha*$_2$-subtype. NG 108-15 *alpha*$_2$-receptors appear to be functionally coupled to adenylate cyclase in an inhibitory manner, confirming previous reports (1), and PAC, like clonidine (1), acts as a partial agonist in inhibiting adenylate cyclase activity (Figs. 9 and 10).

In general, the sites on NG 108-15 cell membranes labeled by [^{3}H]EPI and [^{3}H]PAC are very similar to rat and bovine brain *alpha*$_2$-receptors labeled by the same ligands and also by [^{3}H]clonidine (11, 13), with respect to saturation characteristics (Figs. 2 and 4), pharmacological properties (Table 1), and guanine nucleotide effects (Fig. 5). More specifically, in both tissues, [^{3}H]EPI and [^{3}H]PAC over low concentration ranges label a single population of receptors (Figs. 1–4) which exhibit high affinity for catecholamines and imidazolines (IC_{50} values 1–10 nM). In addition, both agonists and antagonists compete at [^{3}H]EPI and [^{3}H]PAC sites in a homogenous, noncooperative manner in both NG 108-15 membranes, as shown here, and brain membranes (11, 13, 21). Thus in both tissues these ligands under the appropriate experimental conditions appear to bind to one state of the *alpha*$_2$-receptor which has high affinity for agonists and is GTP-sensitive. The similarities between the pharmacological profiles and nucleotide regulation of brain and NG 108-15 *alpha*$_2$-receptors labeled by [^{3}H]PAC and [^{3}H]EPI provide further evidence that brain and NG 108-15 *alpha*$_2$-receptors may be coupled to adenylate cyclase in a similar manner.

When the range of [^{3}H]EPI or [^{3}H]PAC concentrations in saturation experiments is extended beyond those represented in Figs. 2 and 4, Scatchard plots of these data become curvilinear, so that at high concentrations of ^{3}H-labeled agonist, a site with low agonist affinity is demonstrable. This is similar to the situation previously reported in platelets (18).

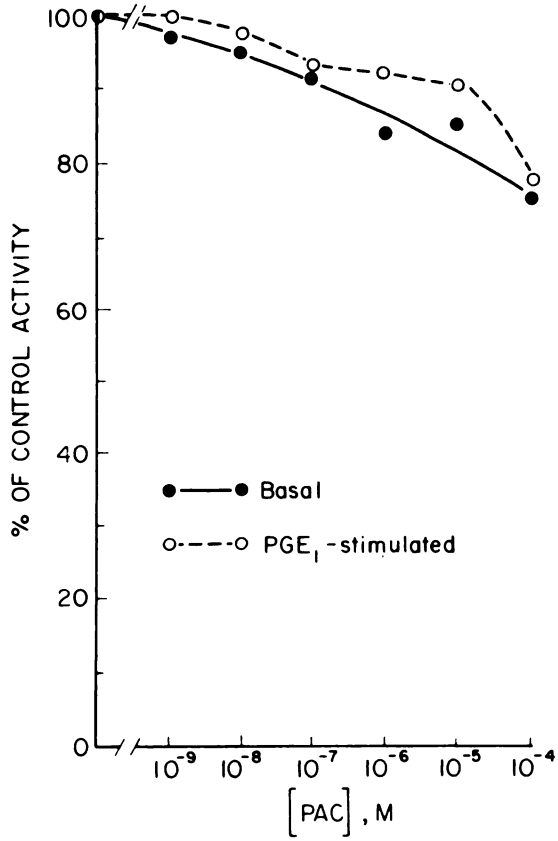

FIG. 9. *Inhibition of basal and PGE_1-stimulated adenylate cyclase activity by PAC*

The effects of increasing concentrations of PAC on basal and PGE_1-stimulated adenylate cyclase activities at 37° are expressed as percentage of control activity measured in the absence of PAC, as described under Materials and Methods (mean values from four experiments). Control values correspond to a mean basal activity of 14.7 pmoles of cyclic [^{32}P]AMP/mg/min, and a PGE_1-stimulated activity of 99.0 pmoles of cyclic [^{32}P]AMP/mg/min.

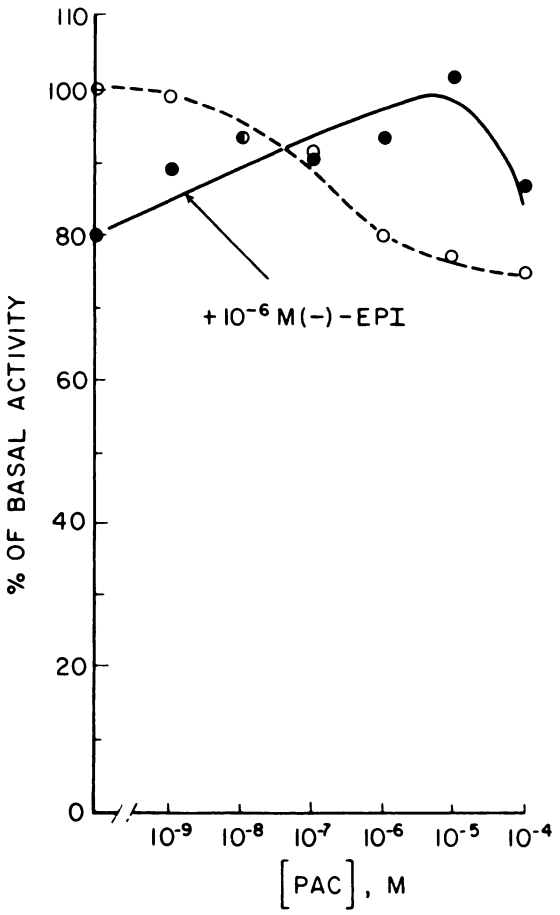

FIG. 10. *Inhibition of basal adenylate cyclase activity and antagonism of the inhibitory effect of 1.0 μM (−)-EPI by PAC*

The effects of increasing concentrations of PAC on basal (○– – –○) adenylate cyclase activity and on inhibition of basal activity caused by 1.0 μM (−)-EPI (●– – –●) at 37 for 10 min are expressed as percentages of control activity in the absence of either drug. Control values correspond to a mean basal activity of 13.9 pmoles of cyclic [^{32}P]AMP/mg/min. Values represent means of four experiments, each performed in duplicate.

The antagonist [^{3}H]YOH is an effective *alpha*$_2$-specific radioligand in NG 108-15 membranes, as previously shown in human platelets (17), and the use of this ligand allows for direct comparisons between *alpha*$_2$-agonist and *alpha*$_2$-antagonist binding. The differences between interactions with NG 108-15 *alpha*$_2$-receptors of [^{3}H]EPI and [^{3}H]PAC on the one hand, and [^{3}H]YOH on the other hand, are analogous to those seen with *beta*-receptor agonist and antagonist radioligand interactions (22–25) and strongly suggest that the *alpha*$_2$-receptor, like the *beta*-receptor, exists in two or more states differentiated mainly with respect to agonist affinities. Thus in NG 108-15 membranes, agonists have much lower affinities at [^{3}H]YOH sites than at [^{3}H]EPI or [^{3}H]PAC sites (Table 1), and in addition exhibit heterogeneous interactions with [^{3}H]YOH sites (Fig. 7), whereas [^{3}H]YOH sites are homogeneous with respect to the radioligand itself (Figs. 5 and 6) and antagonist competitors (Fig. 7). Accordingly, [^{3}H]YOH may label the entire population of high- and low-affinity states of the *alpha*$_2$-receptor [α_2(H) plus α_2(L)] with the same affinity whereas [^{3}H]EPI and [^{3}H]PAC appear to label with high affinity only the α_2(H) fraction of the total *alpha*$_2$-receptor population (cf. ref. 8). The α_2(L) fraction can be observed at very high [^{3}H]EPI and [^{3}H]PAC concentrations, but the variability of the data at these concentrations precludes rigorous analysis.

Surprisingly, some antagonist (YOH, WB-4101, and prazosin) were more potent inhibitors of [^{3}H]YOH binding than of [^{3}H]EPI or [^{3}H]PAC binding. We have observed this tendency when comparing the pharmacological profiles of the three radioligand binding sites in human platelets (17, 20) and rat brain (18). The reasons for this anomaly are not clear at present. Conceivably the state of the NG 108-15 *alpha*$_2$-receptor which has high affinity for agonists [α_2(H)] may exhibit lower affinity for some antagonists.

In brain and platelet membranes (11, 20) and in NG 108-15 membranes in the present study, [^{3}H]PAC appears to label the same high-affinity conformation of the *alpha*$_2$-receptor as the full agonist [^{3}H]EPI, yet PAC is only a partial agonist at NG 108-15 *alpha*$_2$-receptors (Fig. 10). Kent *et al.* (26) observed a discontinuity in an otherwise strong correlation between intrinsic activity and occupancy of high-affinity states of frog erythrocyte *beta*-receptors, in that in the presence of the very weak partial agonist soterenol (10% of the intrinsic activity of isoproterenol) 50% of the *beta*-receptors were still in the high-affinity state. The same model may also apply for the interaction of [^{3}H]PAC with the NG 108-15 *alpha*$_2$-receptor; that is, [^{3}H]PAC labels the same high-affinity state of the receptor as does [^{3}H]EPI, even though it is only a partial agonist. Since a correlation was observed between intrinsic activity and the percentage of the total *beta*-receptor population that was observed to be in the high-affinity state (26), one would predict that [^{3}H]EPI may label more high-affinity *alpha*$_2$-sites than [^{3}H]PAC. This is the case in NG 108-15 cells (see saturation data under Results), and a similar difference is noted between the number of [^{3}H]EPI and [^{3}H]clonidine *alpha*$_2$-receptor sites in brain membranes (21). The number of [^{3}H]YOH sites was found to be greater than the number of [^{3}H]EPI sites on NG 108-15 membranes, which would be predicted if [^{3}H]YOH labeled both α_2(H) and α_2(L), whereas [^{3}H]EPI labeled selectively α_2(H).

A comparison of IC_{50} values of catecholamine agonists in inhibiting the antagonist [^{3}H]YOH binding and in inhibiting adenylate cyclase activity in NG 108-15 membranes suggests that the binding/cyclase potency ratio, K_D/K_{INH}, is about 1.0, whereas the same ratio at ^{3}H-labeled agonist sites is about 0.01. Although the high-affinity, nucleotide-sensitive form of the receptor that is labeled by agonist ligands is believed to be a prerequisite for coupling to adenylate cyclase (8), the affinities of agonists at the antagonist binding site, which in our membrane preparations represent average values for different receptor states, appear to correspond much more closely to the observed potencies of agonists for the adenylate cyclase response. This may be attributable to the fact that agonist potencies for the response reflect the nucleotide-induced *process* of conversion, from high- to low-affinity receptor states, which catalyzes the response (27). The K_D/K_{INH} ratio for clonidine and PAC (using [^{3}H]YOH values) is about 0.1, and probably re-

flects less efficient coupling due to the partial agonist nature of these drugs.

Another tissue which exhibits adenylate cyclase inhibition in response to *alpha*$_2$-receptor stimulation is human platelet (2). Interesting differences exist between binding of [^{3}H]EPI and [^{3}H]PAC in platelets as compared with NG 108-15 cells or brain. High-affinity binding of either ligand is Mg^{2+}-dependent in platelets (20), and to a lesser extent in NG 108-15 cells, but not in brain tissue. Mg^{2+} exerts a permissive role on the guanine nucleotide-induced reduction in high-affinity agonist interactions at the platelet receptor labeled by [^{3}H]PAC (20), [^{3}H]YOH (18), or [^{3}H]dihydro-α-ergocryptine (28). However, in brain membranes Mg^{2+} actually antagonizes GTP effects (6, 29), and in both brain and NG 108-15 membranes low (10 μM) concentrations of GTP increase the number of high-affinity agonist-labeled sites, especially when Mg^{2+} is present. These differences may indicate that the involvement of divalent cations in *alpha*$_2$-receptor-effector coupling mechanisms is somewhat different in neural and non-neural tissues. Alternatively, the level of sequestered endogenous Mg^{2+} and other ions may differ in membranes prepared from different tissues. Regardless of these differences, most *alpha*$_2$-adrenergic receptor systems examined thus far appear to mediate an inhibition of adenylate cyclase, and it is reasonable to speculate this may be the common biochemical response to *alpha*$_2$-stimulation in all tissues, including brain.

There have been two very recent reports of NG 108-15 *alpha*$_2$-receptor labeling using other radioligands, [^{3}H] dihydro-α-ergocryptine (30) and [^{3}H]clonidine (31). Similar GTP-induced shifts in agonist affinities were observed at [^{3}H]dihydro-α-ergocryptine sites, whereas [^{3}H] clonidine binding, with 10 mM Mg^{2+} present, was directly inhibited by guanine nucleotides. In addition, Atlas and Sabol (31) also found PAC to be a partial agonist. Although the data in these studies correspond closely with our observations, one difference was that [^{3}H]clonidine appeared to label both high- and low-affinity forms of the receptor when [^{3}H]clonidine concentrations in saturation experiments were extended to values appreciably greater than labeled agonist concentrations routinely used here.

NG 108-15 cells represent a relatively homogeneous system derived from neural tissue which possesses a pure population of *alpha*$_2$-adrenergic receptors and a well-defined effector response of adenylate cyclase inhibition. Therefore, these cells constitute an excellent model for examining neural receptor-effector coupling mechanisms. The availability of a range of *alpha*$_2$-specific radioligands from full agonist through partial agonist and antagonist should further help to answer fundamental questions concerning the intrinsic differences between agonist and antagonist binding, as well as the cellular mechanisms of regulation of *alpha*$_2$-receptor-mediated responses in the brain and other tissues.

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