MOLECULAR PHARMACOLOGY, 21:600–608

Sodium Inhibits Both Adenylate Cyclase and High-Affinity ^{3}H-Labeled *p*-Aminoclonidine Binding to *Alpha*$_2$-Adrenergic Receptors in Purified Human Platelet Membranes

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Received June 1, 1981; Accepted January 5, 1982

SUMMARY

The effects of sodium on both the binding characteristics of the *alpha*-adrenergic receptor and the activity of *alpha*-receptor-coupled adenylate cyclase of human platelets have been studied. By using a newly developed, partially purified platelet plasma membrane preparation, we have demonstrated that the partial agonist ligand ^{3}H-labeled *p*-aminoclonidine ([^{3}H]PAC) binds to the *alpha*-receptor in a manner which suggests the existence of two types of sites: a high-affinity, low-capacity site ($K_D \equiv 0.87$ nM; $B_{max} \equiv 0.294$ pmole/mg of protein) and a low-affinity site, the properties of which could not be precisely defined. Physiological concentrations of sodium ion decreased the B_{max} for the high-affinity component of [^{3}H]PAC binding while increasing the K_D of the remaining high-affinity sites. Other monovalent cations also decreased [^{3}H]PAC binding, with the order of potency of $Na^+ \geq Li^+ \geq K^+$. Guanine nucleotides markedly diminished the number of high-affinity [^{3}H]PAC binding sites while increasing the K_D of the remaining sites. In the presence of both sodium ion and guanine nucleotides, a distinct high-affinity site could not be identified. Sodium ion significantly decreased the activity of both basal and prostaglandin D_2-stimulated adenylate cyclase activity in the absence of exogenous guanine nucleotide. In the presence of guanine nucleotide (100 μM GTP) there was no inhibitory effect of NaCl on unstimulated enzyme activity, but NaCl inhibited prostaglandin-stimulated adenylate cyclase. Epinephrine (10 μM) inhibited adenylate cyclase activity only when the enzyme had been stimulated by prostaglandin in the presence of 100 μM GTP. The inhibitory effects of epinephrine and NaCl on the prostaglandin-stimulated enzyme were additive. These results suggest that sodium ion may be acting on the adenylate cyclase complex to modulate the interaction of a guanine nucleotide regulatory protein with both the *alpha*$_2$-adrenergic receptor and the enzyme catalytic subunit.

INTRODUCTION

Alpha-adrenergic receptors have been subclassified into *alpha*$_1$- and *alpha*$_2$-subtypes with the use of both physiological and pharmacological criteria (1). Although relatively little is known about the mechanisms involved in mediating *alpha*$_1$-adrenergic receptor responses, *alpha*$_2$-receptors in several tissues are coupled to adenylate cyclase so that receptor activation inhibits both basal and hormone-stimulated enzyme activity (2). *Alpha*-adrenergic receptor inhibition of adenylate cyclase has been studied most extensively in the platelet, where it has been correlated with platelet secretion and aggregation (3) as well as radioligand binding (3–6).

Alpha-adrenergic inhibition of platelet adenylate cyclase activity is dependent upon GTP and is facilitated by sodium ion (7, 8). Ligand binding assays have provided some insight into the mechanisms by which guanine nucleotides modulate *alpha*-adrenergic function in the platelet. Thus, in competition experiments with the ligand [^{3}H]DHEC,[4] guanine nucleotides decrease the affin-

This research was supported by United States Public Health Service Grants MH-15413 and HL-23088.

[1] Recipient of National Institutes of Health Training Grant 2-T32HL017142-06.

[2] Recipient of National Institutes of Health Research Career Development Award K04HL00236.

[3] Recipient of National Institutes of Health Research Career Development Award HL-00332.

[4] The abbreviations used are: DHEC, dihydroergocryptine; PAC, *p*-aminoclonidine; TES, 2-([tris-(hydroxymethyl)methyl]amino)ethane-

0026-895X/82/030600-09$02.00/0

ities of agonists but not those of antagonists for the *alpha*-receptors (9–11). Computer analysis of these data suggest that, in the absence of added guanine nucleotides, platelet *alpha*-adrenergic receptors exist in both a high- and a low-affinity state (9, 10). Other investigators have obtained similar results with other ligands (12, 13). The interpretation of the binding and adenylate cyclase data is that agonists induce the formation of a complex between the platelet *alpha*-adrenergic receptor and a membrane guanine nucleotide regulatory protein, and that the binding of GTP to this complex results ultimately in the inhibition of the adenylate cyclase and dissociation of the agonist from the receptor (9).

The mechanism by which sodium and other monovalent cations facilitate the *alpha*-adrenergic inhibition of platelet adenylate cyclase (7) is less clear. Although sodium, like guanine nucleotides, decreases the affinity of the receptor for agonists, as determined by ligand binding assay (11), computer analysis of epinephrine competition curves for [^{3}H]DHEC suggest that different mechanisms may be involved. Since sodium decreases the affinity of both the high- and low-affinity states of the platelet *alpha*-adrenergic receptor, the possibility has been raised that sodium may act directly on the receptor itself to effect affinity changes (11).

As an approach to the problem of defining the mechanisms by which monovalent cations modulate *alpha*-adrenergic receptor-mediated inhibition of adenylate cyclase in the human platelet, we have developed a method for obtaining partially purified platelet plasma membranes. With the use of the partial *alpha*-adrenergic agonist [^{3}H]PAC (14, 15) and the partially purified platelet membranes, both a high-affinity (K_D = 0.87 nM) and a much lower affinity state of the platelet *alpha*$_2$-adrenergic receptor were identified. Sodium ion and guanine nucleotides each (and together) reduced both the number and affinity of receptors in the high-affinity state. Furthermore, in this membrane preparation, sodium ion alone also inhibited adenylate cyclase activity. The observation that this effect on adenylate cyclase was sensitive to the presence of GTP suggests that sodium ion may exert its effects on both the receptor and adenylate cyclase by a mechanism which alters the interaction of these components with the guanine nucleotide regulatory protein.

EXPERIMENTAL PROCEDURES

Materials. The ligands [^{3}H]PAC (48.5 or 53.4 Ci/mmole) and [^{3}H]DHEC (30.9 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). The disodium salt of Gpp(NH)p, the Tris salt of GTP, (−)-epinephrine bitartrate, and colchicine were purchased from Sigma Chemical Company (St. Louis, Mo.). Unlabeled PAC was donated by Boehringer-Ingelheim, Ltd. (Ridgefield, Conn.).

Platelet membrane preparation. Platelet concentrates were purchased from the American Red Cross Blood Services (Northeast Region) and used within 24 hr of collection. The binding of ligand [^{3}H]DHEC to platelet membranes was 2-fold greater when platelet concentrates less than 24 hr old (eight experiments) were used as compared with the binding of this ligand to membranes prepared from "outdated" platelets obtained 48 hr after collection (three experiments). Platelets were separated from the plasma and contaminating red cells by centrifugation into a two-step arabinogalactan gradient system (16). The bands containing the platelets were separated from the arabinogalactan gradient materials by dilution in a buffered saline-glucose solution containing citrate (16), and centrifugation at 2,000 × *g* for 15 min at 22°. After resuspension in a buffer containing 10 mM TES/140 mM KCl/2.5 mM glucose (pH 7.4), the platelets were incubated in a similar buffer containing 5 × 10^{-4} M colchicine for 45 min in the dark at 37° (17) and then chilled in the dark at 4° for 1 hr. The chilled platelet suspensions were disrupted by two successive decompressions in an ice-chilled nitrogen bomb (Parr Instrument Company, Moline, Ill.) in a well-ventilated hood after being equilibrated at 1000 psi for 45 min in the chilled bomb chamber prior to each decompression. Colchicine treatment was found to facilitate the disruption of platelets by nitrogen decompression (18). The disrupted platelets were centrifuged at 800 × *g* for 15 min at 4° to remove any unbroken or partially broken platelets. The 800 × *g* supernatants were centrifuged at 22,000 × *g* for 35 min at 4°. The resulting pellets were subjected to subcellular fractionation by ultracentrifugation in 30 g/100 ml to 60 g/100 ml linear sucrose density gradients containing 5 mM EDTA (pH 7.2) (19).

The binding of the *alpha*-adrenergic antagonist [^{3}H]-DHEC and the partial agonist [^{3}H]PAC to platelet subcellular fractions from three paired preliminary experiments is shown in Fig. 1. The highest specific binding of these two ligands was found in Fractions 2 and 3, both of which are known to contain platelet membrane vesicles lacking entrapped platelet organelles (19–21). The combined Fractions 2 and 3 contained approximately 4% of the total platelet protein in the suspensions of washed platelets (prior to homogenization), and these fractions had a 20-fold higher specific [^{3}H]DHEC binding activity than did whole platelets at a similar concentration of [^{3}H]DHEC (3). The platelet membrane fractions used here also possessed a 7-fold greater binding activity for [^{3}H]DHEC than did crude particulate preparations (obtained from homogenized platelets) which we have examined previously (3). The specific binding of [^{3}H]DHEC or [^{3}H]PAC to platelet subcellular fractions enriched in platelet subcellular organelles such as mitochondria and platelet granules (Fractions 4–8) was considerably less than that observed for each ligand in Fractions 2 and 3 containing platelet membrane vesicles.

Platelet membranes were collected as a well-defined zone near the top of the gradient ("fractions 2 and 3," ref. 19). After dilution with cold TES/KCl/glucose buffer, the platelet membranes were washed free of the gradient material by centrifugation at 135,000 × *g* for 1 hr at 4° (19). The washed membranes were resuspended in 25 mM TES/5 mM $MgCl_2$ neutralized to pH 7.6 with KOH, quick-frozen in a dry ice/ethanol bath, and stored at −65° for periods of up to 5 days prior to use.

sulfonic acid; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PGD_2, prostaglandin D_2.

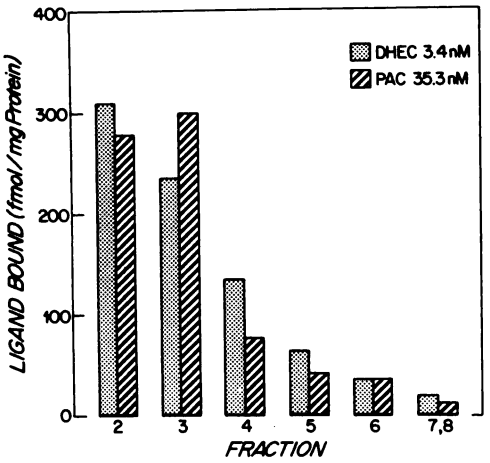

FIG. 1. *Distribution of binding sites for [^{3}H]PAC and [^{3}H]DHEC in human platelet subcellular fractions*

The distribution of mean specific ligand binding activities is shown for the *alpha*-adrenergic antagonist [^{3}H]DHEC (3.4 nM) and the partial *alpha*-adrenergic agonist [^{3}H]PAC (35.3 nM) in human platelet subcellular fractions from three paired experiments. The platelet subcellular fractions were prepared by sucrose density gradient ultracentrifugation (see Experimental Procedures). The numerical designations for the individual platelet subcellular fractions correspond to those previously described (19), and Fractions 2 and 3 contain platelet membrane vesicles which do not have entrapped platelet organelles (19, 20). Buffer II was used as the assay medium.

Radioligand binding assays. Preliminary experiments demonstrated that at 22° the binding of [^{3}H]PAC (5.8 nM) reached equilibrium within 10 min, with a $t_{1/2}$ of about 1 min. Thus, platelet membranes (100 μl) were incubated with [^{3}H]PAC or [^{3}H]DHEC for 20 min at 22° in a covered shaking bath. The final volume was 150 μl and typically contained 30 μg of protein. In most experiments, the assay buffer contained 25 mM TES/5 mM $MgCl_2$ neutralized to pH 7.6 with KOH (Buffer I). Since the pK_a of TES is 7.50, Buffer I contained approximately 13 mM potassium ion. In other experiments, the assay buffer contained 25 mM TES/5 mM $MgCl_2$/135 mM NaCl/1 mM EGTA neutralized to pH 7.6 with NaOH (Buffer II). In each experiment, the reaction was stopped by flooding the tubes with 5 ml of cold assay buffer. Bound and free ligand were separated by filtration through Whatman GF/C glass-fiber filters using a single-port manifold system as previously described (3). Specific binding was defined as the amount of radioactivity displaced by 100 μM (−)-epinephrine, and amounted to 65–95% of total bound radioactivity over the concentration range 0.4–28.0 nM [^{3}H]PAC in Buffer I. In Buffer II, specific binding amounted to 60–70% of total bound radioactivity at a concentration of 6 nM of either [^{3}H]PAC or [^{3}H]DHEC.

In order to show that [^{3}H]PAC was not modified during the binding assay, the ligand was analyzed by thin-layer chromatography after being incubated with platelet membranes under standard assay conditions. Chromatography in ethyl acetate, ethanol, diethylamine (90:8:2, v, v, v) yielded a single peak of radioactivity with an R_F identical with that of the original [^{3}H]PAC.

As described above, after exposure to colchicine, the platelet membranes were well-washed by centrifugation first at 22,000 × *g* and then at 135,000 × *g*. The saturation curves for both [^{3}H]DHEC and [^{3}H]PAC using platelet membranes prepared in the absence of colchicine were similar to those obtained from platelet membranes derived from colchicine-treated homogenates, and Scatchard analysis of such [^{3}H]PAC binding experiments yielded curvilinear plots similar to those obtained from platelet membranes which were treated with colchicine prior to nitrogen decompression.

Agonist-antagonist competition curves were carried out in the presence and absence of 130 mM sodium ion using [^{3}H]DHEC and unlabeled PAC. Membranes were incubated as described above with approximately 5 nM [^{3}H]DHEC and increasing concentrations of unlabeled PAC (10^{-10} M to 10^{-4} M).

Adenylate cyclase assay. Adenylate cyclase activity was measured by the method of Salomon *et al.* (22), as previously described (3), using [α-^{32}P]ATP as substrate and directly measuring the α-^{32}P-labeled cyclic AMP produced. Assay mixtures (final volume 50 μl) contained [α-^{32}P]ATP (1.5×10^6 dpm), 1.0 mM ATP, 25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM cyclic AMP, 0.1% albumin, 10 mM theophylline, 1 mM EGTA, and an ATP-regenerating system consisting of 20 mM creatine phosphate and creatine phosphokinase (1 mg/ml). Reactions were initiated by the addition of 10 μl of membrane suspension containing 20–50 μg of protein and incubated at 37° for 10 min. The reaction was stopped by the addition of 100 μl of 34 mM sodium dodecyl sulfate, 40 mM ATP, and 12 mM cyclic AMP. Data were expressed as picomoles of cyclic AMP formed per milligram of protein per minute.

Protein assay. Protein was determined by the method of Lowry *et al.* (23); bovine serum albumin was used as standard.

Data analysis. The binding data were analyzed by nonlinear least-squares curve fitting to Scatchard plots (nonlinear regression program BMDP-PAR; ref. 24), using a model for ligand receptor interaction which provides estimates for the equilibrium dissociation constants (K_D) and total binding concentrations (B_{max}) in the presence of one ligand and one or two types of binding sites (25). The data for [^{3}H]PAC fit a two-site model significantly better than a one-site model. Within each of eight experiments using [^{3}H]PAC as ligand, the asymptotic standard deviation for the estimated high-affinity site K_D was 17% of the mean (range 5%–31%), whereas the asymptotic standard deviation of the estimated high-affinity site B_{max} was 13% of the mean (range 3%–22%). Whether measured by using either (−)-epinephrine (10^{-4} M; 25 experiments) or phentolamine (10^{-6} M; 2 experiments) to displace [^{3}H]PAC, the asymptotic standard deviations for the K_D and the B_{max} of the low-affinity site were always many times (6- to 2500-fold) greater than the estimated mean values for these two parameters. Consequently, the binding characteristics of this low-affinity site for [^{3}H]PAC could not be defined with precision. In contrast with [^{3}H]PAC, the *alpha*-adrenergic antagonist ligand [^{3}H]DHEC gave a linear plot with a single class of binding sites (K_D = 9.2 nM; B_{max} = 0.960 pmole/mg of

protein) when the data were analyzed by the nonlinear least-squares regression program described above.

The "slope factor" (26) of PAC competition curves with [^{3}H]DHEC was determined by plotting the log of the fraction of specific binding [log ($\bar{y}$/1−$\bar{y}$)] versus the log of PAC concentration, and analyzing the resulting linear plots by least-square regression analysis.

Unless indicated, the data are expressed as group means ± 1 SEM, and groups are compared with each other by using either the two-tailed paired *t*-test or the two-tailed *t* statistic for two means. In other experiments, the concentrations of agonists, partial agonists, or antagonists which inhibited 50% of the binding of [^{3}H]PAC to platelet membranes (the IC_{50}) were determined as previously described (3).

RESULTS

Catecholamines competed with [^{3}H]PAC for binding to the platelet membrane fraction with the potency order of (−)-epinephrine > (−)-norepinephrine > (−)-isoproterenol (Table 1). Binding was stereospecific, since in each case the (−)-stereoisomers were more potent than the (+)-isomers. The *alpha*-adrenergic antagonist phentolamine (IC_{50} = 48 nM) was much more potent in competing for binding than was the *beta*-adrenergic antagonist (±)-propranolol (IC_{50} = 65,000 nM). Thus, the binding site identified by [^{3}H]PAC had the characteristics of an *alpha*-adrenergic receptor. The fact that yohimbine (IC_{50} = 80 nM) is highly potent in competing for [^{3}H]PAC binding suggests that the receptor is of the *alpha*$_2$-subtype.

The effects of sodium ion on the ability of adrenergic agonists to compete for[^{3}H]PAC binding are also shown in Table 1. In the presence of 130 mM NaCl, the IC_{50} for (−)-epinephrine increased from 16 nM to 800 nM, a change of 50-fold. Similar changes were also observed for (−)-norepinephrine. In contrast, the effects of sodium on the competition of *alpha*-adrenergic antagonists for [^{3}H]-PAC binding were minimal (1.5- to 2-fold; Table 1).

TABLE 1

Inhibition of [^{3}H]PAC binding by alpha-adrenergic agonists and antagonists in the presence and absence of 130 mM NaCl

	IC_{50}[a]	
	−Na$^+$	+Na$^+$
	nM	
Agonists and partial agonists		
(−)-Epinephrine	16	800
(±)-Clonidine	32	1,120
(−)-Norepinephrine	80	17,600
(+)-Epinephrine	376	26,000
(+)-Norepinephrine	6,450	72,000
(−)-Isoproterenol	17,648	800,000
(±)-Phenylephrine	ND	192,000
Antagonists		
Phentolamine	48	80
Yohimbine	80	120
Propranolol	65,000	264,000

[a] The concentrations of agonists, partial agonists, or antagonists which inhibit 50% of the specific binding of [^{3}H]PAC to platelet membranes (the IC_{50}) were determined as previously described (3). The concentrations of [^{3}H]PAC in these experiments ranged from 4.5 to 8.1 nM. Each value is the mean of triplicate determinations from two or three separate platelet membrane preparations. ND, Not done.

Specific binding of [^{3}H]PAC to platelet membranes was a saturable process over the concentration range of 0.4–28.0 nM as depicted in Fig. 2, which summarizes eight experiments performed in the absence of sodium ion. Scatchard analysis of each experiment gave Scatchard plots which were resolved into a high-affinity, low-capac-

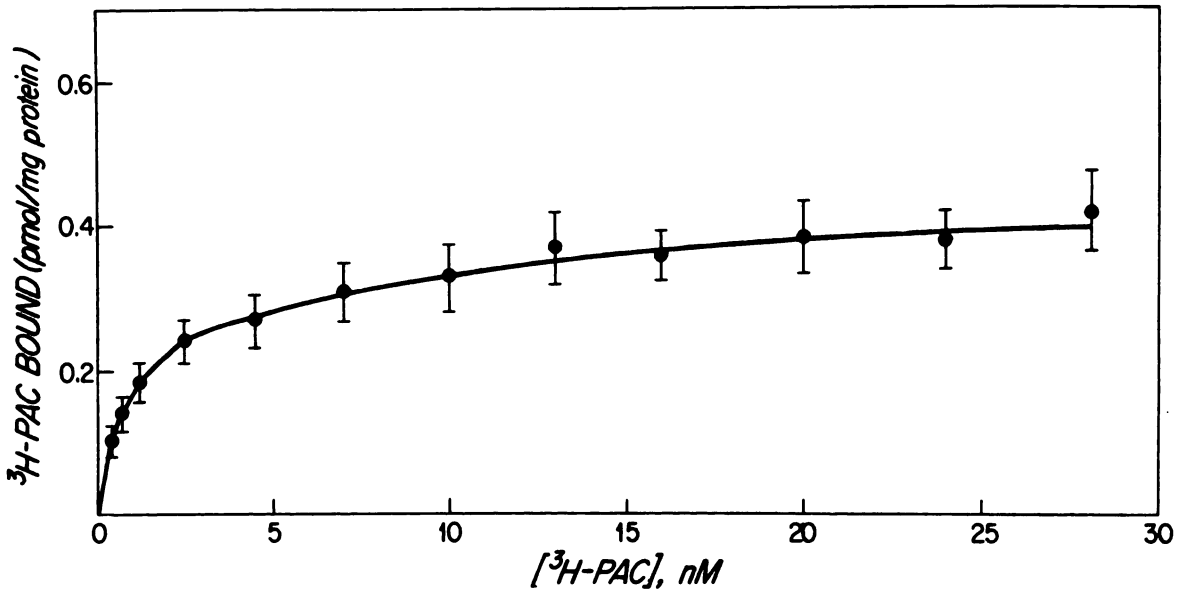

FIG. 2. *Binding of [^{3}H]PAC to platelet membranes*

The mean values from eight separate experiments (± 1 SEM) are shown, using the assay conditions described under Experimental Procedures. The concentration of [^{3}H]PAC varied from 0.4–28.0 nM, and each experiment was performed in Buffer I.

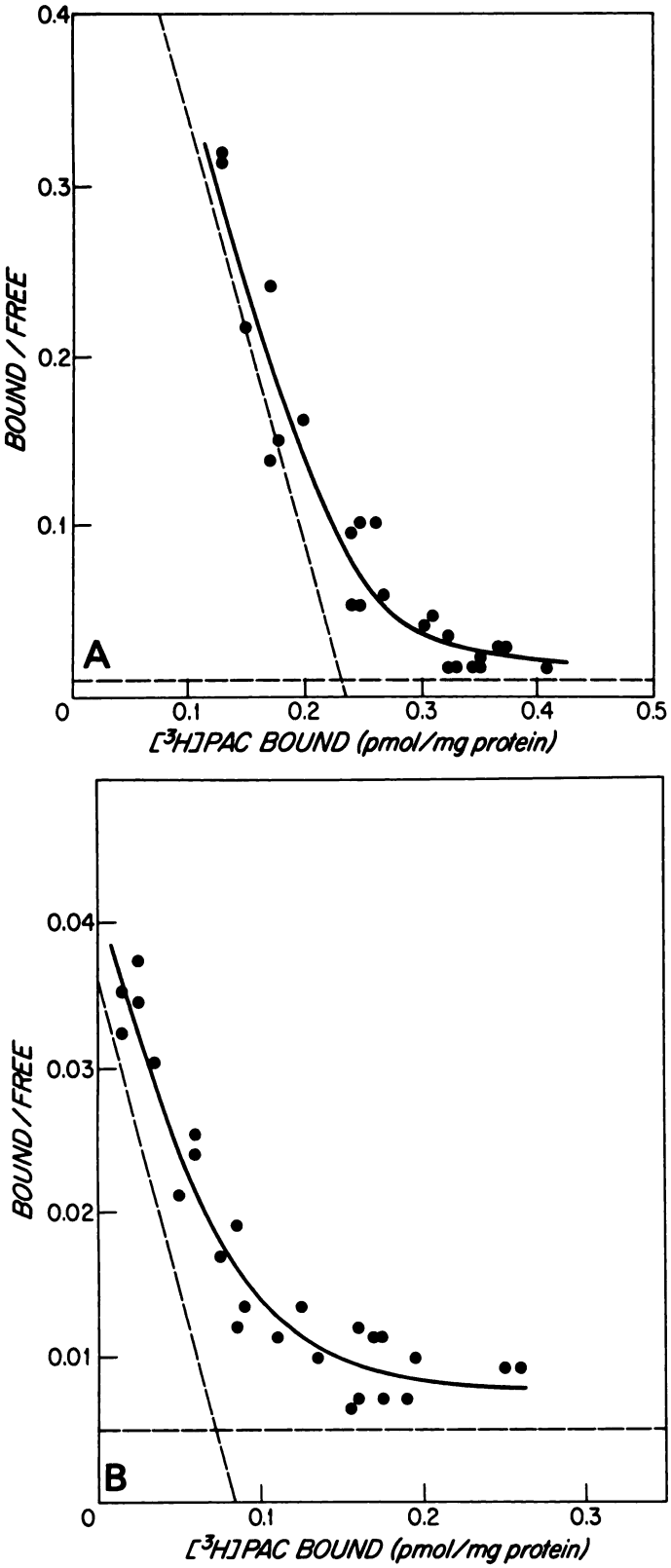

FIG. 3. *Effects of NaCl on binding of [^{3}H]PAC to platelet membranes*

Scatchard analysis of [^{3}H]PAC saturation curves is shown. The concentration of [^{3}H]PAC ranged from 0.4 to 28.0 nM. The curve (——) represents the best-fit regression line as determined by a nonlinear regression technique (see Experimental Procedures). The computer-derived asymptotes of the high- and low-affinity components are indicated by – – –. A, An experiment in which platelet membranes were exposed to [^{3}H]PAC in Buffer I (containing no sodium ion); B, an experiment in which samples of the same membrane preparation were exposed to [^{3}H]PAC in Buffer I containing 130 mM NaCl. When comparing A and B, note the differences in scale in the Scatchard plot axes. With the use of the nonlinear regression program described under Experimental Procedures, the K_D and B_{max} for the high-affinity site in the absence of sodium (A) were 0.4 nM and 0.235 pmole/mg of protein, respectively. In the presence of sodium (B), the K_D and B_{max} for the high-affinity site were 2.3 nM and 0.083 pmole/mg of protein, respectively.

ity site (see Table 2) and a very low-affinity site with a capacity which could not be defined with precision because of a large asymptotic standard deviation (see Experimental Procedures). A typical Scatchard plot from one of these eight experiments is shown in Fig. 3A.

In the presence of sodium ion (130 mM), the binding of [^{3}H]PAC was also saturable, and Scatchard analysis gave a curvilinear plot in each of four experiments. However, computer-assisted analysis by nonlinear least-squares curve fitting of the Scatchard plot demonstrated striking quantitative changes in the high-affinity binding sites at the higher sodium concentration. As shown in Table 2, the number of high-affinity [^{3}H]PAC binding sites was reduced by 64% from 0.294 pmole/mg of protein to 0.106 pmole/mg of protein ($p < 0.01$), whereas the affinity was reduced 10-fold as reflected by the increase in K_D from 0.87 nM to 8.9 nM ($p < 0.02$). A typical Scatchard plot of [^{3}H]PAC binding in the presence of sodium ion is illustrated in Fig. 3B.

Although 130 mM KCl significantly decreased the number of high-affinity binding sites by 58% ($p < 0.01$), this monovalent cation did not significantly change the K_D of the remaining high-affinity [^{3}H]PAC binding sites (Table 2).

Initial experiments had shown that the effects of sodium ion were maximal at concentrations of 100 mM and above. In order to provide further insight into the effects of monovalent cations on [^{3}H]PAC binding, parallel saturation experiments were performed in which NaCl (130 mM), KCl (130 mM), and LiCl (130 mM) were compared. The order of potency in the inhibition of binding of [^{3}H]PAC was $Na^+ > Li^+ > K^+$. Inhibition of [^{3}H]PAC binding was also observed with 200 mM NH_4Cl. When sucrose (250 mM) was substituted for the added cations, binding

TABLE 2

Regulation by monovalent cations of [^{3}H]PAC binding to the high-affinity site of the platelet alpha-adrenergic receptor

Experimental condition	No. of experiments	K_D[a]	B_{max}[a]
		nM	*pmoles ligand bound/mg protein*
Buffer only	8	0.87 ± 0.10	0.294 ± 0.038
Buffer + NaCl (130 mM)	4	8.9 ± 3.8 ($p < 0.02$)	0.106 ± 0.016 ($p < 0.01$)
Buffer + KCl (130 mM)	5	1.35 ± 0.25 (NS)	0.123 ± 0.016 ($p < 0.01$)

[a] The data are expressed as means ± 1 SEM. The K_D and B_{max} of the high-affinity [^{3}H]PAC receptor of platelet membranes in Buffer I alone are compared with the K_D and B_{max} of the high-affinity [^{3}H]PAC receptor of platelet membranes exposed to sodium ion or potassium ion using the two-tailed t statistic for two means (see Experimental Procedures). NS, Not significant ($p > 0.06$).

results were similar to those seen in the absence of added monovalent cations, thus suggesting that the effects of the added monovalent cations cannot be explained on the basis of the changes in osmolarity.

Both GTP and its nonhydrolyzable analogue Gpp (NH)p were found to reduce the affinity of [^{3}H]PAC binding to an equivalent extent, and this effect was maximal at a guanine nucleotide concentration of 100 μM. Although the curvilinear shape of the Scatchard plot was maintained in the presence of GTP (data not shown) and both high- and low-affinity sites were detected, striking quantitative differences were observed in the high-affinity site when compared with the results in the absence of guanine nucleotides with the same membrane preparation. Table 3 shows the results of a series of experiments examining the effects of 100 μM GTP (Tris salt) on [^{3}H]PAC binding. The number of high-affinity sites was significantly reduced by 84% from 0.294 pmole/mg of protein to 0.046 pmole/mg of protein ($p < 0.002$), whereas the K_D was significantly increased from 0.87 nM to 2.8 nM ($p < 0.02$) (Table 3).

Since high-affinity [^{3}H]PAC binding sites were still detectable in the presence of either 100 μM GTP or 130 mM NaCl, experiments were performed to evaluate the combined effects of these compounds on [^{3}H]PAC binding. Computer analysis revealed only a single low-affinity, high-capacity site (data not shown). Thus sodium ion and guanine nucleotides act in an additive manner to eliminate the high-affinity [^{3}H]PAC binding site. In contrast, neither sodium ion nor guanine nucleotides affected the receptor density or the K_D for the *alpha*-adrenergic antagonist [^{3}H]DHEC (data not shown).

As an alternative approach to characterizing the changes in receptor properties induced by sodium ion, competition studies were carried out using [^{3}H]DHEC and unlabeled PAC in the absence and in the presence of 130 mM NaCl. The specific binding of [^{3}H]DHEC in the presence of increasing concentrations of PAC (10^{-10} M to 10^{-4} M) was expressed as a percentage of total specific binding ($\bar{y}$), and the log ($\bar{y}/1-\bar{y}$) plotted against the log[PAC] (see Fig. 4). In the absence of sodium ion, the results indicated a value of 0.08 μM for the IC_{50} and a "slope factor" of 0.47, consistent with the presence of more than one class of binding sites with different affinities. In the presence of 130 mM NaCl, the value of the IC_{50} increased to 0.34 μM, and the slope factor increased to 0.81, indicating that some, but not all, of the high-affinity binding sites had been converted to a lower affinity state, a result consistent with the direct analysis of [^{3}H]PAC binding described above.

The inhibition of adenylate cyclase by unlabeled PAC and (−)-epinephrine were compared in order to assess the intrinsic activity of PAC, since the binding characteristics of the tritiated ligand suggested that it was acting as an agonist. Lysates were prepared by freezing and thawing a pellet of washed platelets and resuspend-

TABLE 3

Regulation by guanine nucleotides of [^{3}H]PAC binding to the high-affinity site of the platelet alpha-adrenergic receptor

Experimental condition	No. of experiments	K_D[a]	B_{max}[a]
		nM	*pmoles ligand bound/mg protein*
Buffer only	8	0.87 ± 0.10	0.294 ± 0.038
Buffer + GTP (100 μM)	4	2.8 ± 0.93 ($p < 0.02$)	0.046 ± 0.011 ($p < 0.002$)
Buffer + GTP (100 μM) + NaCl (130 mM)	4	—[b]	—[b]

[a] The data are expressed as means ± 1 SEM. The K_D and B_{max} of the high-affinity [^{3}H]PAC receptor of platelet membranes in Buffer I alone as presented in Table 2 are compared here with the K_D and B_{max} of the high-affinity receptor component of platelet membranes exposed to guanine nucleotides using the two-tailed t statistic for two means (see Experimental Procedures).

[b] High-affinity [^{3}H]PAC receptor binding sites were not detected by nonlinear regression analysis in the presence of both sodium ion and guanine nucleotides.

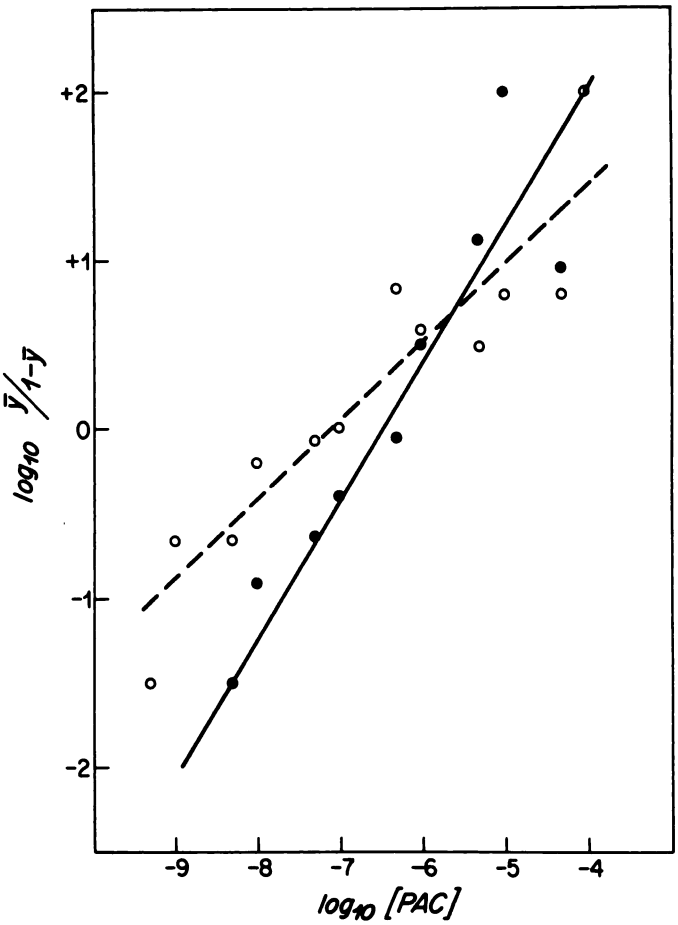

FIG. 4. *Effects of NaCl on the inhibition by PAC of [^{3}H]DHEC binding to platelet membranes*

The binding of [^{3}H]DHEC (5 nM) in the presence of increasing concentrations of unlabeled PAC (10^{-10} M to 10^{-4} M) was measured in the presence and in the absence of 130 mM NaCl. Total specific binding of [^{3}H]DHEC, determined by measuring [^{3}H]DHEC binding in the absence of unlabeled ligand and correcting for the nonspecific binding in the presence of 10^{-4} M (−)-epinephrine, was the same in the absence and in the presence of sodium ion. The "slope factor" (26) and IC_{50} were calculated by plotting the log of the fraction of specific binding [log ($\bar{y}/1-\bar{y}$)] versus the log of PAC concentration and analyzing the resulting linear plots by least-square regression analysis. The data points are the means of two experiments performed in duplicate. The values of the "slope factor" and IC_{50} in the absence of sodium ion (○; $r = 0.93$) were 0.47 and 0.08 μM, respectively. The values of the "slope factor" and IC_{50} in the presence of 130 mM NaCl (●; $r = 0.94$) were 0.81 and 0.35 μM, respectively.

ing the lysed platelets in 25 mM Tris-HCl buffer (pH 7.4). In addition to the tissue and the assay "cocktail" (see Experimental Procedures), assay tubes contained 10 μM PGD_2 and either (−)-epinephrine or PAC (10^{-9} M to 10^{-4} M). The results were compared with the activity of samples containing only PGD_2. The results of three experiments examining the inhibition of PGD_2-stimulated adenylate cyclase by PAC are shown in Fig. 5. Epinephrine inhibited 70% of the adenylate cyclase activity stimulated above basal by PGD_2, with an IC_{50} of approximately 0.5 μM. PAC inhibited 40% of the stimulated activity with an IC_{50} of about 0.2 μM. Thus, PAC had an intrinsic activity of about 60% (40%/70% × 100) of that of (−)-epinephrine.

Since sodium ion has been reported to facilitate the coupling of inhibitory receptors to adenylate cyclase (27), the effect of 130 mM NaCl on the coupling of the *alpha*-adrenergic receptor to adenylate cyclase was investigated in this membrane preparation. The results, compared by paired *t*-test, are presented in Table 4. Unstimulated adenylate cyclase activity was measured as described under Experimental Procedures in the presence or in the absence of 100 μM GTP, and either 10 μM epinephrine or 130 mM NaCl, or epinephrine and NaCl together. Likewise, the effects of either 10 μM epinephrine or 130 mM NaCl, or epinephrine and NaCl together on PGD_2-stimulated enzyme activity were determined both in the presence and in the absence of 100 μM GTP. The mean basal adenylate cyclase activity of seven platelet membrane preparations was 7.9 ± 1.3 pmoles/mg of protein per minute (Table 4). In the absence of exogenous GTP, sodium ion decreased the level of basal enzyme activity in each experiment, with a mean value for the seven experiments of 6.1 ± 1.3 pmoles/mg of protein per minute ($p < 0.04$). In contrast, in the presence of 100 μM GTP (adenylate cyclase activity = 11.3 pmoles/mg of protein per minute) no inhibition by NaCl was observed. Epinephrine (10 μM) had no effect on the unstimulated adenylate cyclase activity under any of these conditions.

The inhibitory effect of sodium was even more pronounced when adenylate cyclase was activated by PGD_2 (10 μM). In the absence of exogenous GTP, NaCl decreased the activity from 48.1 ± 9.1 pmoles/mg of protein per minute to 28.5 ± 5.4 pmoles/mg of protein per minute ($p < 0.01$). When 100 μM GTP was present, NaCl reduced the activity from 112.7 ± 17.8 pmoles/mg of protein per minute to 95.4 ± 17.4 pmoles/mg of protein per minute ($p < 0.01$), an amount similar to the inhibition of the stimulated enzyme by NaCl in the absence of GTP. Epinephrine alone had no effect on hormone-stimulated adenylate cyclase activity in the absence of exogenous GTP. However, when 100 μM GTP was present, epinephrine reduced the activity of the stimulated enzyme from 112.7 ± 17.8 pmoles/mg of protein per minute to 86.4 ±

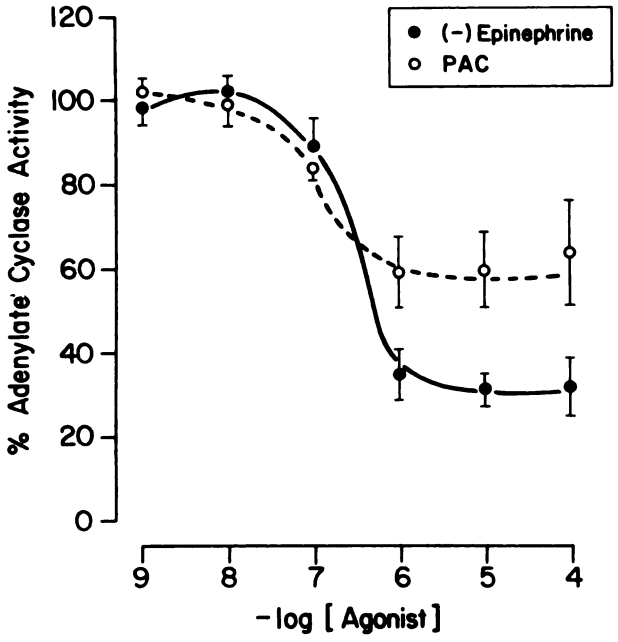

FIG. 5. *Inhibition of PGD_2-stimulated platelet adenylate cyclase by PAC and (−)-epinephrine*

In three experiments, adenylate cyclase activity was assayed by using freeze-thawed preparations of washed normal platelets according to the method of Salomon *et al.* (22). In tubes containing platelet lysate and "assay cocktail," the basal activity was 2.2 pmoles of cyclic AMP per milligram of protein per minute. To assess the stimulation of adenylate cyclase activity by PGD_2, platelet lysate was added to tubes containing assay cocktail and 10 μM PGD_2. The mean 100% PGD_2-stimulated activity was found to be 89.9 pmoles of cyclic AMP per milligram of protein per minute. Alpha-adrenergic-mediated inhibition of PGD_2-stimulated adenylate cyclase was examined by the addition of platelet lysate to tubes containing assay cocktail, 10 μM PGD_2, and either (−)-epinephrine or PAC (10^{-9} M to 10^{-4} M). The data are expressed as mean percentages of the PGD_2-stimulated cyclase activity ± 1 SD.

TABLE 4

Inhibition of platelet membrane adenylate cyclase by sodium ion or (−)-epinephrine

	Adenylate cyclase activity[a]	Significance[b]
	pmoles/mg protein/min	
No addition	7.9 ± 1.3	
+ Epinephrine (10 μM)	7.8 ± 1.5	NS
+ NaCl (130 mM)	6.1 ± 1.3	$p < 0.04$
+ Epinephrine + NaCl	5.4 ± 1.0	$p < 0.01$
GTP (100 μM)	11.3 ± 1.7	
+ Epinephrine (10 μM)	11.3 ± 1.2	NS
+ NaCl (130 mM)	11.5 ± 1.7	NS
+ Epinephrine + NaCl	9.9 ± 1.0	NS
Prostaglandin D_2 (10 μM)	48.1 ± 9.1	
+ Epinephrine (10 μM)	42.5 ± 6.8	NS
+ NaCl (130 mM)	28.5 ± 5.4	$p < 0.01$
+ Epinephrine + NaCl	29.8 ± 5.7	$p < 0.01$
Prostaglandin D_2 + GTP	112.7 ± 17.8	
+ Epinephrine (10 μM)	86.4 ± 11.0	$p < 0.02$
+ NaCl (130 mM)	95.4 ± 17.4	$p < 0.001$
+ Epinephrine + NaCl	71.6 ± 12.1	$p < 0.001$

[a] The data represent the mean ± 1 SEM of the results of seven experiments. A different membrane preparation was used for each experiment. In each experiment, a single membrane preparation was used for assaying activity under all experimental conditions.

[b] Because of the range in enzyme activities of individual membrane preparations (basal activity = 3.6 to 13.0 pmoles/mg of protein per minute), the effects of epinephrine, NaCl, and the combination of the two agents were compared with the relevant control (no additions, GTP, prostaglandin alone, or prostaglandin + GTP) using a paired *t*-test (see Experimental Procedures). NS, Not significant ($p > 0.05$).

11.0 pmoles/mg of protein per minute ($p < 0.02$). The inhibitory effects of sodium ion and epinephrine upon the prostaglandin-stimulated enzyme activity in the presence of 100 μM GTP were additive.

DISCUSSION

In this study, we have examined the effects of sodium on binding of the partial agonist [^{3}H]PAC to *alpha*-adrenergic receptors and on *alpha*-adrenergic inhibition of adenylate cyclase. In these membranes, [^{3}H]PAC gives a curvilinear Scatchard plot which is resolvable into a high-affinity, low-capacity site and a low-affinity site. Like guanine nucleotides, monovalent cations reduce markedly the number of receptors in the high-affinity state. Sodium also reduces both basal and hormone-stimulated levels of adenylate cyclase activity. This inhibition is modulated by guanine nucleotides in a complex fashion.

The partially purified platelet membrane preparation used in these experiments offers three significant advantages over the crude particulate preparations which we (3) and others (4–6, 9–13) have used previously to study platelet *alpha*-adrenergic receptors. First, the other particulate preparations contain membranes from various intracellular structures such as mitochondria and granules. In contrast, with use of the procedure for platelet subcellular fractionation described above, platelet plasma membranes are concentrated in Fractions 2–3, which do not contain granules or mitochondria (19, 21). Second, the density of *alpha*-adrenergic receptors in the partially purified platelet membrane fraction (as measured by [^{3}H]DHEC binding) is 5- to 7-fold greater than that reported previously in other studies (3, 4). Finally, the partially purified platelet plasma membranes appear to contain relatively little endogenous nucleotide, since little or no *alpha*-adrenergic inhibition of adenylate cyclase is seen in this membrane preparation without the addition of guanine nucleotides.

The ligand used here, PAC, differs somewhat from the parent compound clonidine in its interaction with platelet *alpha*-receptors. First, the affinity of [^{3}H]PAC for platelet receptors is higher than that of [^{3}H]clonidine (12). More important, [^{3}H]PAC binding to platelet membranes is more sensitive to regulation by guanine nucleotides than is [^{3}H]clonidine. Thus, the high-affinity state of the platelet *alpha*-adrenergic receptor identified by [^{3}H]PAC is almost completely abolished by guanine nucleotides whereas [^{3}H]clonidine binding is decreased only 20% by 100 μM GTP (12). This sensitivity of [^{3}H]PAC to regulation by guanine nucleotides probably reflects the relatively high intrinsic activity of PAC. In the formulation of Hoffman *et al.* (9), the ability to induce the formation of a complex between the receptor and a membrane guanine nucleotide regulatory protein is a fundamental property of an agonist. Further substantiating the proposition that the binding properties of [^{3}H]PAC reflect its properties as a partial agonist is the contrast with the antagonist ligand [^{3}H]DHEC, which in the same membrane preparation gave a linear Scatchard plot which was not affected by guanine nucleotides or cations.

The high-affinity, low-capacity [^{3}H]PAC binding site seen in the absence of added guanine nucleotides probably represents the high-affinity state resulting from the agonist-induced formation of a receptor-guanine nucleotide regulatory protein complex (9). In the experiments measuring the binding of [^{3}H]PAC, the binding parameters of the low-affinity site cannot be quantitated with precision, presumably because of its very low affinity. This low-affinity receptor state may represent those receptors that are uncoupled from the nucleotide regulatory protein. The sum of receptors in this state and the high-affinity receptors should equal the number of receptors detected with the antagonist ligand [^{3}H]DHEC, which does not distinguish between high- and low-affinity *alpha*$_2$-adrenergic receptors (10). Our inability to quantitate the number of low-affinity receptors precludes a direct demonstration using [^{3}H]PAC of conversion by guanine nucleotides (and monovalent cations) from high- to low-affinity receptors, although this remains the most likely explanation for the nucleotide-induced decrease in the high-affinity binding.

In contrast with the effects of guanine nucleotides on [^{3}H]PAC binding which could be predicted from the work of others, the effects of monovalent cations on [^{3}H]PAC binding and adenylate cyclase activity in this membrane preparation provide new insights into the role of sodium in *alpha*$_2$-adrenergic inhibition of platelet adenylate cyclase. Thus, sodium by itself is capable of markedly reducing the number of receptors in the high-affinity binding state, as shown by a direct estimation of the *alpha*$_2$-adrenergic receptor density determined by the binding of [^{3}H]PAC. The striking changes in "slope factor" and K_D calculated from PAC-[^{3}H]DHEC competition curves (Fig. 4) provide evidence that high-affinity binding sites are being converted to a low-affinity state by Na^+.

The ability of Na^+ to destabilize the agonist-induced high-affinity receptor state suggested that the cation is modifying an interaction between the receptor and a nucleotide regulatory protein. The data on Na^+ inhibition of adenylate cyclase suggest that the cation also modifies the interaction between the nucleotide regulatory protein and the catalytic unit. The modulation of the inhibitory effect of Na^+ on adenylate cyclase by guanine nucleotides (Table 4) provides additional evidence that the sodium effect is mediated through the regulatory protein.

The most detailed previous analysis of the effects of sodium on agonist interaction with platelet *alpha*-adrenergic receptors suggested that sodium decreased agonist affinity by decreasing the affinity of both high- and low-affinity states without changing the proportion of receptors in the two states (11). The difference between the effects of sodium ion and the effects of guanine nucleotides on high-affinity binding led to the suggestion that sodium and nucleotides acted at different sites with sodium perhaps acting at the receptor itself, since the low-affinity state presumably is not coupled to the regulatory protein (11). The inhibition of both basal and prostaglandin-stimulated adenylate cyclase activity by sodium ion in the absence of hormone (Table 4) suggests that the action of sodium ion upon the *alpha*-adrenergic receptor-adenylate cyclase complex cannot be explained solely by the effects of sodium ion upon the receptor. Although multiple sites of interaction of sodium ion with the several individual components of the platelet *alpha*-adrenergic receptor-adenylate cyclase complex cannot be ex-

cluded, the binding data and adenylate cyclase data presented here are fully consistent with a single effect of sodium ion upon a guanine nucleotide regulatory protein which in turn influences the relationships between the regulatory protein and both the *alpha*-adrenergic receptor and the adenylate cyclase catalytic unit. The action of monovalent cations upon a nucleotide regulatory protein is supported by the recent report of Steer and Wood (28) that monovalent cations slow the activation of platelet adenylate cyclase by Gpp(NH)p. Also Aktories *et al.* (29) have recently postulated a direct interaction of cations with the guanine nucleotide regulatory protein of the adenylate cyclase complex of the hamster adipocyte. Thus, there is growing evidence to support the suggestion that sodium may exert its effects on receptor binding and adenylate cyclase activity by acting on the guanine nucleotide regulatory protein. The computer modeling of the effects of Na^+ on agonist displacement curves of [^{3}H]DHEC, which show a decrease of affinity of both low- and high-affinity receptors, suggests that Na^+ may also act upon the receptor itself (11).

In summary, we have provided detailed evidence that, in human platelets, sodium and other monovalent cations both modify high-affinity *alpha*$_2$-adrenergic agonist binding and inhibit adenylate cyclase activity. Our data are consistent with the possibility that sodium acts at a site on or associated with inhibitory guanine nucleotide regulatory proteins to alter the interaction of the regulatory proteins with both the *alpha*-adrenergic receptor and the adenylate cyclase catalytic unit. The use of agonist ligands and the purified plasma membrane preparations should facilitate the elucidation of the molecular mechanisms involved in these processes.

ACKNOWLEDGMENTS

We are grateful to Cindy Boiardi and Andrea Todaro for their excellent technical assistance, to Dr. Donald O'Hara for very helpful discussions concerning the data analysis, and to the Hospital Services Department of the American Red Cross Blood Services (Northeast Region), Boston, Mass. 02115.

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