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Sodium Ion Modulates Agonist and Antagonist Interactions with the Human Platelet *Alpha*$_2$-Adrenergic Receptor in Membrane and Solubilized Preparations

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SUMMARY

Monovalent cations are known modulators of hormonal inhibition of adenylate cyclase. The present studies examine the effects of monovalent cations on human platelet *alpha*$_2$-adrenergic receptors coupled to inhibition of adenylate cyclase. Studies of (−)-epinephrine competition for [^{3}H]yohimbine antagonist binding to human platelet membranes indicate that Na^+ decreases *alpha*$_2$-receptor affinity for agonists by more than one order of magnitude in a manner that is synergistic with the effects of guanine nucleotides. This Na^+-induced decrease in *alpha*$_2$-receptor affinity for agonists is also reflected in the ability of Na^+ to decrease, in a concentration-dependent manner, the quantity of [^{3}H]-epinephrine binding to membranes that is detectable with vacuum filtration assays. In contrast to effects of *alpha*$_2$-receptor agonist interaction, Na^+ slightly increases both *alpha*$_2$-receptor affinity for the antagonist, [^{3}H]yohimbine, and the apparent density of binding sites for this radioligand. Ionic strength in the incubations was maintained constant using *N*-methyl-D-glucamine as the sodium substitute. Despite the qualitatively different effects of Na^+ on *alpha*$_2$-receptor-agonist and *alpha*-$_2$-receptor-antagonist interactions, the similar specificity of monovalent cations (i.e., $Na^+ > Li^+ > K^+$) and the EC_{50} for Na^+ (i.e., 5–15 mM) in mediating these interactions suggest that a common Na^+ binding site might mediate both effects. Sensitivity of *alpha*-receptor-ligand interactions to monovalent cations is retained in digitonin-solubilized preparations whether or not the *alpha*-receptor is solubilized unoccupied or occupied by agonist or antagonist agents at the time of solubilization. In contrast, the sensitivity of *alpha*$_2$-receptor-agonist interactions to guanine nucleotides is retained only when *alpha*$_2$-receptors are occupied by the [^{3}H]-epinephrine agonist at the time of solubilization [Smith, S. K., and L. E. Limbird. *Proc. Natl. Acad. Sci. U. S. A.* **78**:4026–4030 (1980)]. These data suggest that guanine nucleotide and sodium modulation of the *alpha*$_2$-adrenergic system is mediated by different molecular components.

INTRODUCTION

A variety of hormones and drugs have been demonstrated to lower basal and hormone-stimulated cyclic AMP levels. It has been postulated that the resulting decrement in cyclic AMP levels may mediate, at least in part, the physiological effects induced by these agents and provide a mechanism for countering the effects mediated by elevation of cyclic AMP concentrations with the same target organ (1). Hormonal attenuation of cyclic AMP levels in intact cells has been demonstrated to result from decreased synthesis of cyclic AMP rather than from stimulation of cyclic AMP degradation (2). Direct effects of hormones on inhibition of adenylate cyclase activity in broken-cell preparations appear to require GTP and, in most target membranes, to be enhanced by sodium ion (1). In addition, the effects of GTP and Na^+ on inhibitory adenylate cyclase systems are manifest not only on catalytic activity but also on receptor-ligand interactions (3), suggesting that the regulatory moieties conferring sensitivity to nucleotides and monovalent cations interact with both the receptor and catalytic subunits of the inhibitory adenylate cyclase system.

In hormonal systems coupled to activation of adenylate cyclase, significant understanding of the sequence of molecular events involved in receptor-cyclase coupling emerged from determining which interactions among the components of the adenylate cyclase system were stabilized by the biological effectors themselves, namely hormones (or agonist drugs) and guanine nucleotides, and could therefore be isolated as molecular complexes sub-

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sequent to detergent solubilization (4–8). A similar approach has been applied to the *alpha*$_2$-adrenergic system of human platelets coupled to inhibition of basal and prostaglandin E_1-stimulated adenylate cyclase activity and has demonstrated that agonist occupancy of the *alpha*$_2$-receptor stabilizes receptor interactions with membrane effector components, one of which is likely the GTP-binding protein modulating *alpha*-receptor affinity for agonist agents (9, 10). This conclusion is based on two independent observations. First, agonist occupancy of the *alpha*-receptor prior to digitonin solubilization promotes formation of an agonist-*alpha*$_2$-receptor complex of molecular size larger than unoccupied or antagonist-occupied *alpha*$_2$-receptors, reflected by its faster sedimentation in sucrose gradients (9, 10). Second, this agonist *alpha*$_2$-receptor complex retains its sensitivity to guanine nucleotides, manifested by the guanine nucleotide-facilitated dissociation of [^{3}H]epinephrine from the prelabeled, solubilized receptors (10). These data suggest that monitoring the sensitivity of solubilized receptors or receptor-ligand complexes to modulators of receptor function may provide a convenient assessment of whether or not the effector component(s) which mediate the effects of these agents remains associated with the receptor under a particular experimental condition.

The goal of the present studies was to extend the above observations exploring the molecular basis for hormonal inhibition of adenylate cyclase and thus characterize the effects of sodium and other monovalent cations on *alpha*-receptor-agonist and *alpha*-receptor-antagonist interactions in the human platelet membrane. Furthermore, we assessed whether or not sensitivity to monovalent cations was retained in digitonin-solubilized preparations in an effort to learn whether or nor the *alpha*-adrenergic receptor itself might be useful as an "affinity adsorbent" for isolation of the component conferring sensitivity to Na^+ and analysis of its function in subsequent reconstitution studies.

METHODS

Materials. ^{3}H-Labeled (−)-epinephrine (68 Ci/mmole) and [^{3}H]yohimbine (81 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). Phentolamine was a gift from Ciba Pharmaceuticals (Summit, N. J.), and expired platelet concentrates, when used, were provided by the Nashville Chapter of the American Red Cross. The sources of all other materials have been reported previously (10).

Preparation of human platelet membranes. Human platelet membranes from freshly obtained platelet-rich plasma were prepared as described previously (10). Occasionally, membrane preparations were prepared from outdated (3-day-old samples) platelet concentrates stored in acid-citrate-dextrose. Platelets from outdated concentrates were washed one time in 150 mM NaCl, 10 mM Tris-HCl, and 20 mM EDTA (pH 7.65) by centrifugation for 15 min at 2200 rpm. The washed platelets were resuspended to approximately 10 mg/ml in lysing buffer [7.5 mM Tris-HCl and 5 mM EDTA (pH 7.5)] and stored in liquid nitrogen until the day of intended use. The frozen platelets were then thawed and disrupted with a Brinkmann Polytron for two 5-sec bursts at Setting 5, interrupted by 30 sec on ice. Disruption and all subsequent membrane washes were carried out as described for freshly obtained platelets (10). The final membrane preparation was suspended by Teflon-glass homogenization into 75 mM Tris-HCl, 12.5 mM $MgCl_2$, and 1.5 mM EDTA (pH 7.65) (Buffer A).

It should be noted that qualitatively identical effects of monovalent cations were noted in preparations derived from fresh platelets and outdated platelet concentrates. However, only 60–80% of the density of [^{3}H]yohimbine binding sites was found in outdated platelet preparations.

Preparation of solubilized preparations. Membrane pellets obtained by centrifugation of membranes suspended in Buffer A at 39,000 × *g* for 10 min (4°) were resuspended in a 25 mM Tris-HCl, 5 mM $MgCl_2$, and 2.5 mM EDTA buffer (pH 7.8) containing 1% digitonin with 10 up-and-down strokes of a 15-ml Dounce homogenizer, Pestle A. The protein to detergent ratio was approximately 1.5–3.0 mg of membrane protein to 1 ml of digitonin-containing buffer during the homogenization step. The typical concentration of digitonin-solubilized protein in the resultant 100,000 × *g* supernatant was 0.6–1.8 mg/ml in preparations from fresh platelets and intentionally greater in preparations derived from outdated platelets. Solubilization was accomplished by shaking at 15° (prelabeled receptor preparations) or stirring on ice (unoccupied receptors) for 30 min. The preparation was centrifuged at 105,000 × *g* for 60 min and the supernatant from this centrifugation is referred to as the "solubilized preparation."

Assay of radioligand binding. [^{3}H]Yohimbine binding to human platelet membranes was determined by incubation for 30 min at 30° in a final volume of 0.25 ml containing 5–12 nM [^{3}H]yohimbine, 30 mM Tris-HCl, 5 mM $MgCl_2$, 0.6 mM EDTA, 0.25–0.4 mg of membrane protein, and the concentrations of *alpha*-adrenergic competitors and/or monovalent cations noted in the figures and corresponding legends. The incubation was terminated by the addition of 5 ml of ice-cold Buffer A, filtration under vacuum through Whatman G/F C glass-fiber filters, and two additional 5-ml buffer washes. The filters were dried for 30 min at 60° and counted in 10 ml of Triton-toluene fluor. Nonspecific binding of [^{3}H]yohimbine was defined as the quantity of radioligand binding not competed for by 10 μM phentolamine and was 5–15% of total binding.

Prior to assay of [^{3}H]yohimbine binding to digitonin-solubilized *alpha*-adrenergic receptors, the solubilized preparation was exchanged into 0.1% digitonin-containing buffers by chromatography on Sephadex G-50 columns (2.0 × 22 cm) prewashed with 30 ml of 0.1% digitonin in 25 mM Tris-HCl, 5 mM $MgCl_2$, and 2.5 mM EDTA (pH 7.8) and eluted with the same buffer. The volume corresponding to that for elution of Blue Dextran 2000 ("void volume") was collected and utilized for assay of [^{3}H]yohimbine binding to *alpha*-adrenergic receptors unoccupied at the time of solubilization of the human platelet membranes. Incubation to determine [^{3}H]yohimbine binding to solubilized preparations was carried out for 90 min at 15° in a 0.25-ml volume containing 20 mM Tris-HCl, 6 mM $MgCl_2$, 1.6 mM EDTA, and 0.1% digitonin. At the end of the incubation, 0.25 ml of ice-cold

Buffer A containing 0.025% digitonin was added to the incubation tube and the entire 0.5-ml volume was transferred to a Sephadex G-50 column (0.6 × 14 cm) equilibrated and eluted with the 0.025% digitonin-containing buffer. The 0.9-ml volume corresponding to the "void-volume" was collected and counted in 10 ml of Triton-toluene scintillation fluor. From termination of incubation to collection of the total void volume required approximately 6 min. Nonspecific binding, defined as above, was less than 20% of total binding.

As described previously (10), the conditions for determining the binding of [^{3}H]epinephrine to human platelet membranes were modified from those used to study [^{3}H]yohimbine binding in an effort to stabilize the radioligand from oxidation and degradation as well as to decrease nonspecific binding of [^{3}H]epinephrine to the membrane preparation. Thus, incubations of 1 ml containing 0.25–0.4 mg of membrane protein per milliliter were carried out for 2 hr at 15° in a mixture typically containing 6–9 nM [^{3}H]epinephrine, 1 mM pyrocatechol, 0.1% ascorbic acid, 1 μM pargyline, and 1 μM propranolol. The incubation was terminated as for [^{3}H]yohimbine assays. Nonspecific binding, defined as above, was 16–40% of total binding.

Prelabeling of human platelet membranes. Human platelet membranes were prelabeled with 6–12 nM [^{3}H]epinephrine or 12–15 nM [^{3}H]yohimbine under conditions employed for the assay of [^{3}H]epinephrine binding. The prelabeling was terminated by centrifugation at 39,000 × *g* for 10 min at 4°. The prelabeled pellets were then solubilized as described above. To determine the amount of prelabeled radioligand bound to the solubilized *alpha*-adrenergic receptors at any time point, separation of bound from free radioligand in 0.5-ml aliquots was accomplished (at 5°) by chromatography on Sephadex G-50 columns (0.6 × 14 cm) equilibrated and eluted as described above for [^{3}H]yohimbine binding to solubilized preparations.

Choice of a "sodium substitute." To differentiate specific effects of monovalent cations from "nonspecific effects" on receptor-ligand interactions due simply to changes in ionic strength, a "sodium substitute" was used to replace sodium or other monovalent cations in the incubation and thus maintain a constant osmolality in all studies. Choline chloride, even when freshly purified, was deleterious to radioligand binding and adenylate cyclase activities. Tris-HCl, at concentrations greater than 80 mM, had effects of its own, particularly on adenylate cyclase activity. However, *N*-methyl-D-glucamine, neutralized to pH 7.4 with HCl, was without effect on [^{3}H]yohimbine binding, [^{3}H]epinephrine binding, or adenylate cyclase activity in intact membrane as well as digitonin-solubilized preparations and was thus chosen as the sodium substitute for maintaining ionic strength of added monovalent cations at 120 mM in all of the studies described.

RESULTS

Effects of sodium on alpha-receptor-agonist interactions in human platelet membranes. Figure 1 compares the effects of NaCl with those of guanine nucleotides on the affinity of human platelet *alpha*-adrenergic receptors for the agonist, (−)-epinephrine. The reduced receptor affinity induced by both sodium ion and the hydrolysis-resistant GTP analogue, Gpp(NH)p,[2] was manifested by an increase in the EC_{50} for (−)-epinephrine in competing for [^{3}H]yohimbine binding to human platelet membranes when incubations were carried out in the presence of 100 mM NaCl or 0.1 mM Gpp(NH)p. The effects of Na^+ and guanine nucleotides appeared to be synergistic, or at least additive, since the simultaneous presence of both effectors at their maximally effective concentrations promoted a decrease in receptor affinity which was greater than the effect of either modulator alone.

As is discussed below, sodium ion affects not only *alpha*-receptor-agonist but also *alpha*-receptor-antagonist interactions. Thus, it seemed inappropriate to use competition profiles as shown in Fig. 1 to monitor quantitatively the dose-dependent effects of monovalent cations on receptor-agonist interactions, since increases in sodium concentration alter both the quantity of [^{3}H]yohimbine binding detected (i.e., changes in the *y* axis) as well as the potency of (−)-epinephrine as a competitor for this binding (i.e., changes along the *x* axis). However, as reported previously (10, 11), *alpha*-receptor-agonist interactions can be monitored directly in human platelet membrane preparations using [^{3}H]epinephrine as the radioligand. At low concentrations (< 10 nM) of [^{3}H]epinephrine, the radioligand binds almost exclusively to

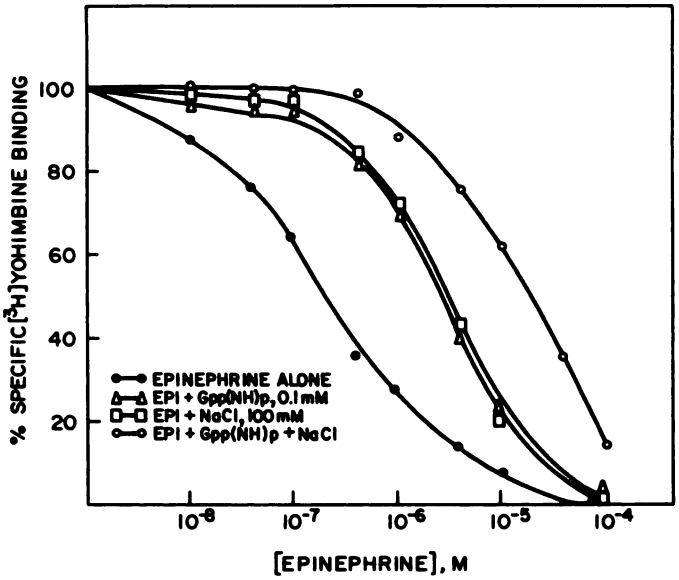

FIG. 1. *Effect of sodium ion and guanine nucleotides on human platelet alpha*$_2$*-receptor-agonist interactions*

Human platelet membranes were incubated with 7.5 nM [^{3}H]yohimbine in a final volume of 250 μl as described under Methods in the presence of increasing concentrations of (−)-epinephrine in the absence or presence of 0.1 mM Gpp(NH)p, 100 mM NaCl, or both. The experiment shown is representative of four separate experiments performed in duplicate. Maximal [^{3}H]yohimbine binding (100% specific binding on the *y* axis) was 3700 cpm (141 fmoles/mg of membrane protein) in the absence of NaCl and 4100 cpm (156 fmoles/mg of membrane protein) in the presence of NaCl. Data are plotted as percentage of control binding obtained in the absence of *alpha*-adrenergic competitor but in the presence of NaCl, Gpp(NH)p, or NaCl ± Gpp(NH)p, when added.

[2] The abbreviation used is Gpp(NH)p, 5′-guanylyl imidodiphosphate.

the high-affinity, guanine nucleotide-sensitive state of the *alpha*-receptor for agonists (10, 11). The reduction in *alpha*-receptor affinity for agonists induced by sodium results in an inability to "trap" lower-affinity *alpha*-receptor-[^{3}H]epinephrine complexes using vacuum filtration techniques to separate bound from free radioligand (12). Consequently, the effects of sodium and other monovalent cations on *alpha*-receptor-agonist interactions can be monitored by their ability to reduce the quantity of [^{3}H]epinephrine binding that is detectable with our current procedures.

Figure 2 demonstrates the specificity of monovalent cations in decreasing the quantity of [^{3}H]epinephrine binding to human platelet membranes that is detected using vacuum filtration assays. In all incubations, the ionic strength was maintained constant using *N*-methyl-D-glucamine as the "inert cation," as described under Methods. Thus, the dose-dependent effects of the monovalent cations cannot be attributed to incremental changes in ionic strength. The order of potency of monovalent cations in decreasing detected [^{3}H]epinephrine binding is $Na^+ > Li^+ > K^+$, and Na^+ has an EC_{50} of approximately 15 mM in decreasing *alpha*-receptor affinity for the agonist epinephrine.

Effect of sodium on alpha-receptor-antagonist interactions in human platelet membranes. In early pilot experiments, we noted that sodium appeared to increase the EC_{50} of antagonists in competing for [^{3}H]yohimbine binding. However, the increase in EC_{50} for phentolamine or yohimbine in competing for [^{3}H]yohimbine binding was small as compared with ion-induced decreases in *alpha*-receptor affinity for agonists. In addition, the observed changes in receptor-antagonist interactions were extremely variable and appeared to correlate with the extent to which total [^{3}H]yohimbine binding was increased by the presence of sodium. We later appreciated that the increased EC_{50} for antagonists observed in competition studies was *not* a manifestation of a sodium-induced decrease in receptor affinity for antagonists, but rather was an artifact resulting from the increased binding of [^{3}H]yohimbine in the presence of sodium and hence a greater quantity of receptor binding for which to compete, thus giving rise to an increased EC_{50} value for antagonist competitors. The increased [^{3}H]yohimbine binding detected in these early competition studies studies was later determined in saturation binding studies to result from a sodium-induced increase both in the density and in the affinity of human platelet *alpha*-adrenergic receptors for [^{3}H]yohimbine. Figure 3A compares the Scatchard transforms of [^{3}H]yohimbine saturation binding data in the presence of 40 mM and 120 mM NaCl. The slight increase in receptor affinity promoted by Na^+ is fully effected at 40 mM concentrations of the monovalent cation, although the sodium-induced increase in *alpha*-receptor density (assessed by the intercept on the *x* axis) is greater at 120 mM NaCl than at 40 mM NaCl. These data suggest that effects on receptor affinity and density for antagonists may be mediated by independent monovalent cation sites. The data in Fig. 3B indicate that the rate of [^{3}H]yohimbine dissociation from human platelet membranes is not modified by Na^+, and thus the slight increase in *alpha*-receptor affinity for antagonists induced by Na^+ appears to be due solely to an accelerated rate of [^{3}H]yohimbine associated in the presence of Na^+ (Fig. 3C). The molecular basis by which monovalent cations might accelerate radioligand association is not known. It should be mentioned that the dissociation of [^{3}H]yohimbine remains monophasic even when dissociation is monitored over a longer period of time than shown in Fig. 3B. Thus, the data in Fig. 3A–C are entirely consisent with the interaction of [^{3}H]yohimbine with a homogeneous class of binding sites.

Effects of sodium ion on alpha-receptor-antagonist interactions in detergent-solubilized human platelet preparations. The *alpha*-adrenergic receptor of human platelets can be solubilized from the human platelet membrane using the plant glycoside digitonin (9, 10). We have previously demonstrated that the solubilized *alpha*-receptor retains its recognition properties for *alpha*-adrenergic agonists and antagonists but loses its sensitivity to guanine nucleotides unless the receptor is occupied by an agonist agent prior to solubilization (9, 10). Thus, it was of interest to determine whether the modulation of *alpha*-receptor-antagonist interactions by sodium ion would be retained by *alpha*-adrenergic receptors subsequent to detergent solubilization. Figure 4A is a Scatchard transform of saturation binding data obtained by incubating *alpha*-receptors, unoccupied by adrenergic agents at the time of solubilization, with [^{3}H]yohimbine subsequent to digitonin solubilization. The effects of Na^+ on saturation binding profiles of solubilized *alpha*-adrenergic receptors are comparable to those observed in the intact membranes. Thus, Na^+ increases both the affinity

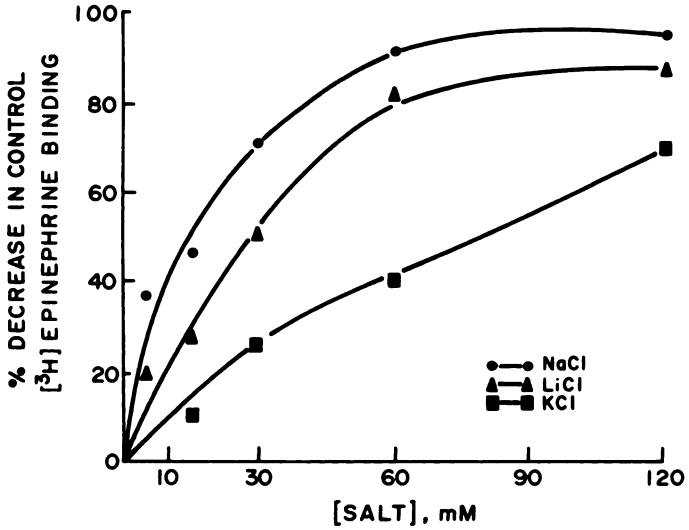

FIG. 2. *Specificity of the effect of monovalent cations on alpha-receptor-agonist interactions*

[^{3}H]Epinephrine binding was carried out as described under Methods in the presence of 0–120 mM concentrations of NaCl, LiCl, or KCl. Ionic strength in all incubations was maintained constant using *N*-methyl-D-glucamine-HCl as the "inert cation." The ability of monovalent cations to decrease the amount of [^{3}H]epinephrine binding detected is plotted as percentage control of [^{3}H]epinephrine binding detected. The data shown are the means of five separate experiments performed in duplicate, where 100% specific ^{3}H-labeled (−)-epinephrine binding varied from 800 to 1400 cpm per incubation (44–70.1 fmoles/mg of membrane protein).

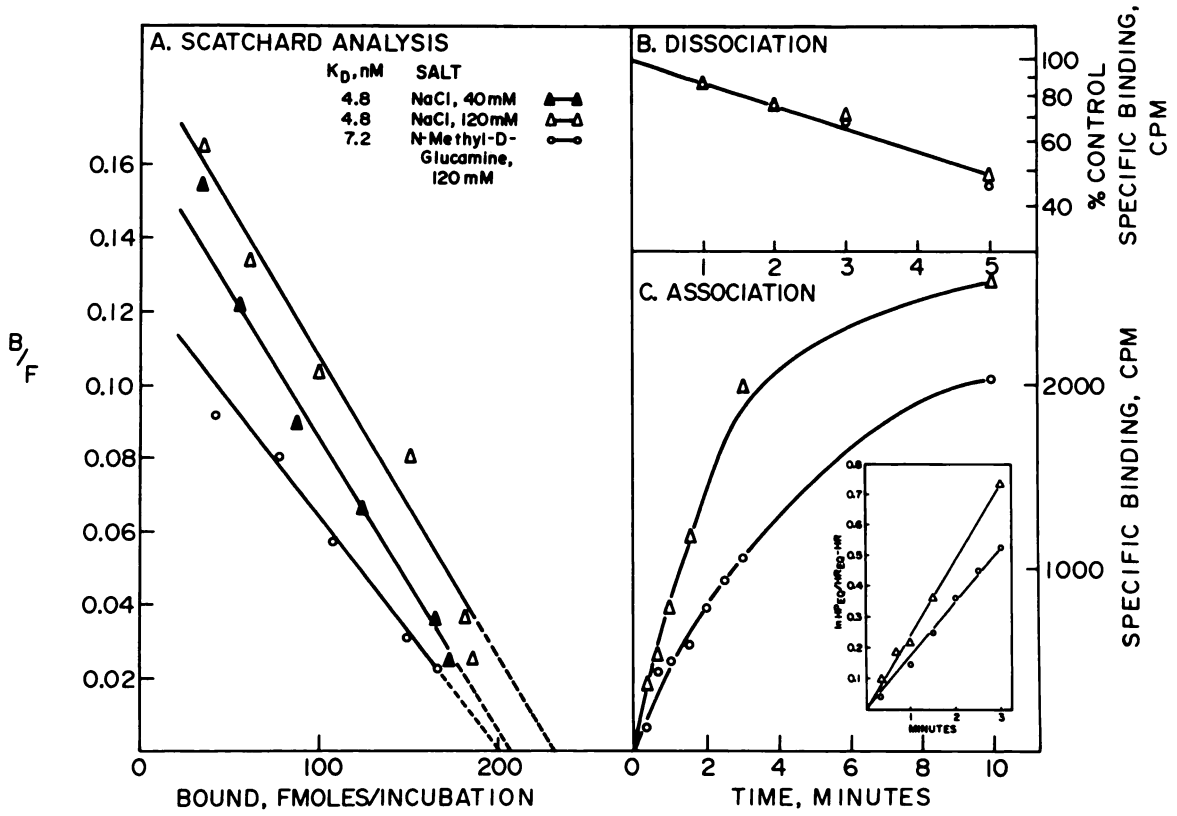

FIG. 3. *Effect of sodium ion on alpha-receptor-antagonist interactions in intact human platelet membranes*

A. Comparisons of the Scatchard transforms of [^{3}H]yohimbine saturation binding data (0.8–32 nM) in the presence of 120 mM *N*-methyl-D-glucamine (control), 40 mM NaCl (+80 mM *N*-methyl-D-glucamine) or 120 mM NaCl. The data are from one experiment representative of three experiments performed in duplicate, where B_{max} was increased by 22 ± 4.5% in the presence of 120 mM NaCl and Na^+-induced increases in receptor affinity were 46–52%. The calculated K_D values were 4.8 nM in the presence of 40 mM and 120 mM NaCl, and 7.2 nM in the presence of *N*-methyl-D-glucamine. The B_{max} values were 149 fmoles/mg of membrane protein for 120 mM *N*-methyl-D-glucamine, 154 fmoles/mg of membrane protein for 40 mM NaCl, and 173 fmoles/mg of membrane protein for 120 mM NaCl. Protein was present at 1.34 mg/250-μl incubation. B_{max} values obtained in the experiment shown using outdated platelets were 60% of those typically obtained using fresh platelets. However, qualitatively similar effects of NaCl in increasing B_{max} values (16–23% increase) were observed in fresh platelet preparations. K_D values for [^{3}H] yohimbine and their modulation by NaCl were essentially identical in preparations derived from fresh versus outdated platelets.

B. Effect of NaCl on the rate of dissociation of [^{3}H]yohimbine from human platelet membranes. Human platelet membranes were incubated with 6.9 nM [^{3}H]yohimbine for 30 min at 30°. The dissociation phase was initiated by the addition of 10 μM phentolamine in the presence of 120 mM NaCl or 120 mM *N*-methyl-D-glucamine (control). The data are plotted as percentage of specific binding observed at time zero immediately subsequent to the 10% dilution effected by the addition of 10 μM phentolamine and salts. The calculated k_{diss} value was 0.139 min^{-1} in the experiment shown. A mean k_{diss} value of 0.146 ± 0.01 min^{-1} was obtained from three separate experiments performed in duplicate.

C. Effect of NaCl on the rate of association of [^{3}H]yohimbine with human platelet membranes. Membranes (1.0 mg of membrane protein per milliliter) were preincubated with other incubation constituents, as described under Methods, for 5 min at 30° prior to the addition of 3.2 nM [^{3}H] yohimbine, which initiated the association phase. At the time points shown, duplicate 250-μl aliquots were rapidly transferred to 5 ml of ice-cold Buffer A and passed over Whatman GF/C filters under vacuum. The filters were then washed two additional times with ice-cold Buffer A. The steady state was typically reached within 10–12 min, although association was studied for 30 min. The *inset* shows the same data transformed so that a k_{ass} value can be obtained from the slope = $k_{observed} = k_{ass}$ [H] + k_{diss}. The value of k_{diss} used for these calculations was 0.145 min^{-1}, as obtained in B. The calculated k_{ass} for the experiment shown was 0.027 nM^{-1} min^{-1} in the presence of NaCl and 0.009 nM^{-1} min^{-1} in the presence of *N*-methyl-D-glucamine. Similar values were obtained in three additional experiments performed in duplicate. Thus, the faster rate of [^{3}H] yohimbine association in these four experiments in the absence of a change in the rate of dissociation (cf. Fig. 3B) would predict a 1.8- to 3.0-fold increase in *alpha*-receptor affinity for [^{3}H]yohimbine in the presence of NaCl, which is in reasonable agreement with the 1.5- to 2.5-fold increase in *alpha*-receptor affinity shown in A and other experiments. The kinetically derived K_D values from four separate association experiments were 3.1 ± 1.2 nM in the presence of NaCl and 9.3 nM ± 4.1 nM (mean ± standard error) in the presence of *N*-methyl-D-glucamine, again in reasonable agreement with the K_D values obtained in steady-state binding experiments.

and capacity of *alpha*-receptor binding sites for [^{3}H] yohimbine. However, in contrast to membrane preparations, Na^+ accelerates both the rate of [^{3}H]yohimbine dissociation from (Fig. 4B) and the rate of association with (Fig. 4C) human platelet solubilized preparations. This effect of Na^+ to facilitate [^{3}H]yohimbine dissociation from human platelets is observed both when receptors are unoccupied at the time of solubilization (Fig. 4B) and when receptors are prelabeled with [^{3}H]yohimbine prior to solubilization (inherent in the conditions shown in Fig. 5).

The ability of Na^+ to facilitate [^{3}H]yohimbine dissociation from solubilized receptor-antagonist complexes provided a convenient monitor of the specificity and potency of monovalent cations in modulating solubilized *alpha*-receptor-antagonist interactions. Thus, as shown

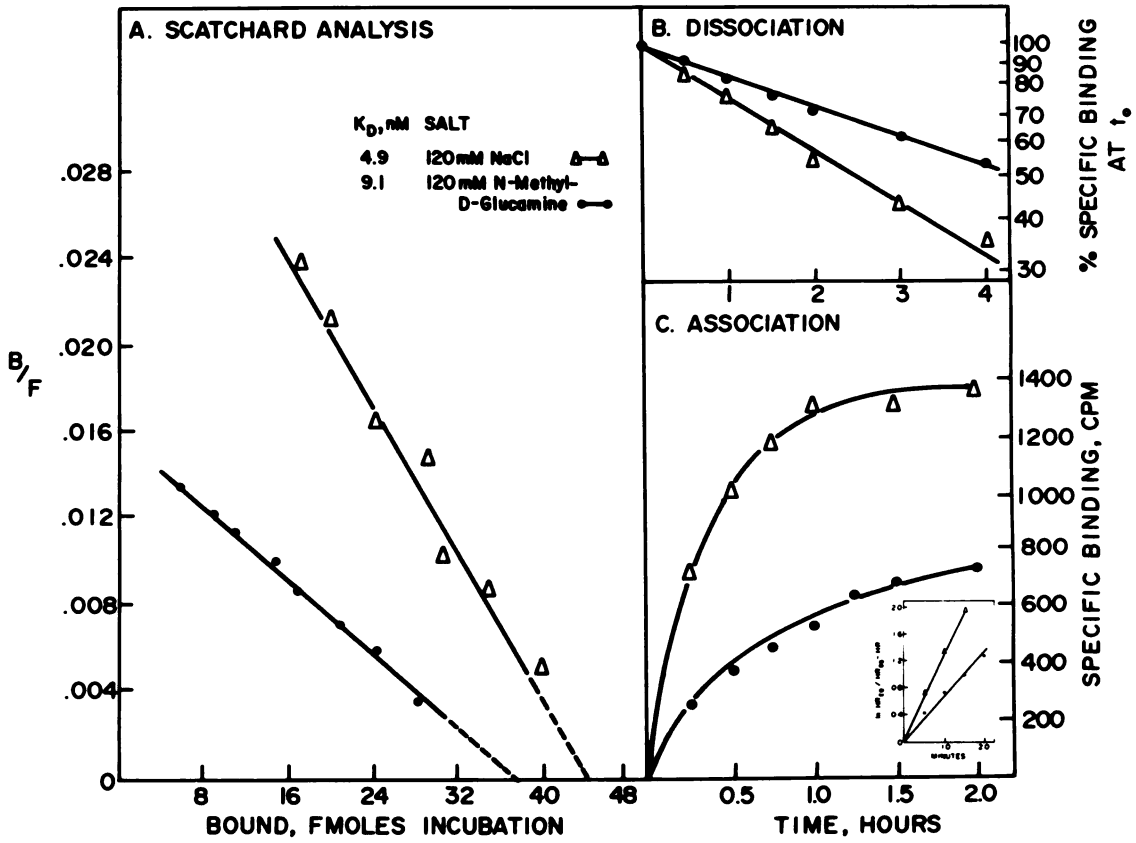

FIG. 4. *Effect of sodium on alpha-receptor-antagonist interactions in digitonin-solubilized human platelet preparations*

A. Scatchard transform of steady-state binding of [^{3}H]yohimbine (1.9–31.0 nM) to digitonin-solubilized preparations. Incubations were performed as described under Methods, except that binding was for 4 hr at 15° to allow the lowest concentrations of radioligand to reach steady state. The K_D value for [^{3}H]yohimbine binding calculated from the slope of the Scatchard plot was 4.9 nM in the presence of 120 mM NaCl and 9.1 nM in the presence of *N*-methyl-D-glucamine. The B_{max} value calculated from the *x*-intercept was 266 fmoles/mg in the presence of *N*-methyl-D-glucamine and 333 fmoles/mg in the presence of NaCl, indicating a 22% increase in binding capacity induced by NaCl. The data shown are from one experiment representative of five separate experiments in which Na^+-induced increases in apparent receptor affinity were 1.9- to 3.3-fold, and Na^+-induced increases in receptor density were 14–25% (20 ± 1.8% SE).

B. Effect of NaCl on the dissociation of [^{3}H]yohimbine from solubilized *alpha*-adrenergic receptors. Membranes were solubilized with 1% digitonin-containing buffers as described under Methods, exchanged into 0.1% digitonin-containing buffers, and incubated with 9.8 nM [^{3}H] yohimbine for 90 min at 15°. The dissociation phase was initiated by adding 10 μM phentolamine with either *N*-methyl-D-glucamine or NaCl to a final concentration of 120 mM. Addition of the *alpha*-adrenergic antagonist and the salts resulted in a 10% dilution of the preparation. Time zero represents binding immediately after the dissociation phase was initiated. The amount of binding at different times was determined by transferring 0.5-ml aliquots to Sephadex G-50 columns as described under Methods. The data shown represent the mean of two separate experiments which varied by less than 5%. The calculated rate of dissociation, k_{diss}, was 0.0027 min^{-1} in the presence of *N*-methyl-D-glucamine and 0.0048 min^{-1} in the presence of NaCl.

C. Effect of NaCl on the rate of [^{3}H]yohimbine association with digitonin-solubilized *alpha*-adrenergic receptors. Solubilized preparations were exchanged into 0.1% digitonin and after a 5-min preincubation at 15°, were exposed to 13 nM [^{3}H]yohimbine, which initiated the association phase. Transfer of the 0.5-ml incubate to a G-50 column to separate bound from free radioligand was assumed to terminate the association phase. The data shown are from one experiment representative of three separate experiments. The k_{ass} calculated from the transform shown in the *inset* was 0.00113 nM^{-1} min^{-1} in the presence of *N*-methyl-D-glucamine and 0.0038 nM^{-1} min^{-1} in the presence of NaCl, indicating a 3.4-fold increase in the rate of association. The calculated K_D (k_{diss}/k_{ass}), based on the dissociation rate values calculated from B, was 2.4 nM in the presence of *N*-methyl-D-glucamine and 1.27 nM in the presence of NaCl. Thus, the nearly 2-fold increase in affinity promoted by Na^+ calculated from the kinetic constants agrees favorably with the steady-state data shown in A, although there was a quantitative difference in the values obtained using steady-state versus kinetic parameters.

in Fig. 5, the percentage of maximal [^{3}H]yohimbine dissociation induced by 120 mM NaCl after incubation for 2 hr at 15° could be compared for increasing concentrations of various monovalent cations. In the studies shown in Fig. 5, the [^{3}H]yohimbine-*alpha*-receptor complexes were formed prior to solubilization, and the rate of dissociation from the prelabeled complexes was studied. Comparable results are obtained with *alpha*-receptor preparations unoccupied at the time of solubilization. The specificity of monovalent cations in facilitating [^{3}H] yohimbine dissociation from solubilized *alpha*-receptors was $Na^+ > Li^+ > K^+$, and the EC_{50} for Na^+ was approximately 5–10 mM. This specificity and affinity for Na^+ is comparable to the observed effects of monovalent cations in modulating *alpha*-receptor-agonist interactions in intact membranes (cf. Fig. 2), except that K^+ appears somewhat more potent in modulating solubilized *alpha*-receptor-antagonist interactions than intact membrane *alpha*-receptor-agonist interactions.

Effects of sodium on alpha-receptor-agonist complexes in solubilized human platelet preparations. Figure 6 compares the ability of both guanine nucleotides

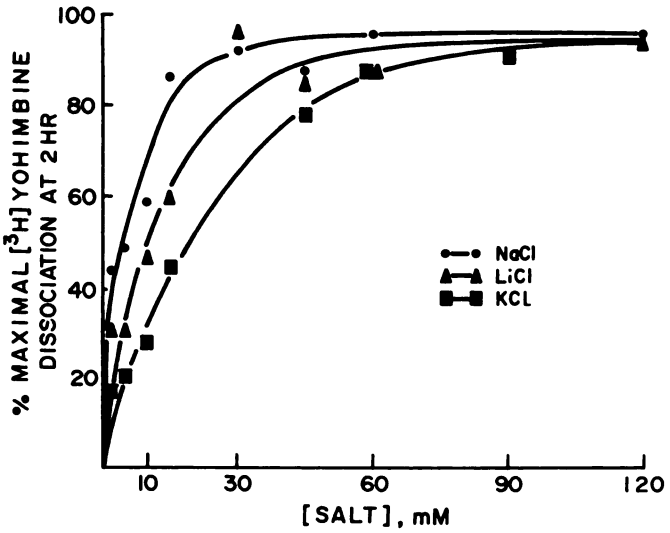

FIG. 5. *Specificity of monovalent cations in facilitating the dissociation of [^{3}H]yohimbine from digitonin-solubilized [^{3}H]yohimbine-alpha-receptor-complexes*

Human platelet membranes prelabeled with 8–14 nM [^{3}H]yohimbine were solubilized with digitonin as described under Methods. At time zero, the dissociation phase was initiated by the addition of 10 μM phentolalamine and the concentration of salts shown, keeping ionic strength constant by the addition of *N*-methyl-D-glucamine. Dissociation proceeded at 15° for 2 hr. The amount of radioligand remaining bound at 2 hr under the various salt conditions was determined by using Sephadex G-50 chromatography. The data are plotted as percentage of maximal [^{3}H]yohimbine dissociation induced by 120 mM NaCl at 2 hr. The maximal additional dissociation induced by NaCl was typically 20–30% (cf. Fig. 4B), and the difference in counts per minute per 0.5-ml aliquot, constituting 100% of the effect of NaCl, was 1100–1700 cpm. The results shown are the mean of five to seven experiments performed in duplicate.

and sodium ion to facilitate [^{3}H]epinephrine dissociation from human platelet *alpha*-receptors in intact membrane (Fig. 6A) and digitonin-solubilized (Fig. 6B) preparations. Both Na^+ and guanine nucleotides alone facilitate [^{3}H]epinephrine dissociation from human platelet membrane *alpha*-receptors, and have a greater effect when present together. These findings are consistent with the observations in competition binding studies presented in Fig. 1. As shown in Fig. 6B, the ability of guanine nucleotides and sodium to facilitate [^{3}H]epinephrine dissociation from the *alpha*-receptor is retained in digitonin-solubilized preparations. Thus, as described previously (10), occupancy of human platelet *alpha*-adrenergic receptors with the agonist [^{3}H]epinephrine prior to exposure to digitonin permits retention of receptor sensitivity to guanine nucleotides subsequent to solubilization. The solubilized [^{3}H]epinephrine-*alpha*-receptor complex also retains its sensitivity to sodium ion, as evidenced by the ability of Na^+ to facilitate [^{3}H]epinephrine dissociation from the prelabeled receptor complex. Furthermore, the concomitant inclusion of Na^+ and Gpp(NH)p at the outset of the dissociation phase results in a faster rate of [^{3}H]epinephrine dissociation from solubilized *alpha*-receptors than observed with either effector alone, in a manner analogous to the apparently synergistic effects of Na^+ and guanine nucleotides in intact membrane preparations (Fig. 1 and Fig. 6A). Both Na^+ and Gpp(NH)p have their more significant effects on the faster phase of the multiphasic [^{3}H]epinephrine dissociation. At present, the molecular basis for the complex dissociation kinetics for [^{3}H]epinephrine or for the effects of Na^+ and Gpp(NH)p predominantly on the more rapidly dissociating phase are not known.

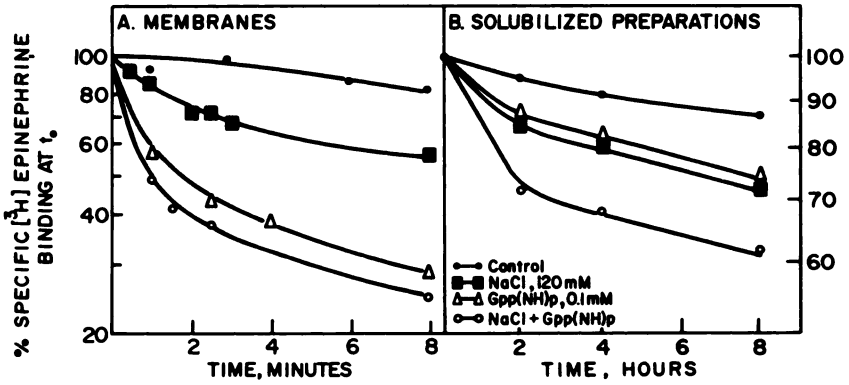

FIG. 6. *Sensitivity of [^{3}H]epinephrine-alpha-receptor complexes to sodium and guanine nucleotides*

A. Dissociation of [^{3}H]epinephrine from human platelet membranes in the presence of 0.1 mM Gpp(NH)p or 120 mM NaCl, or both. Human platelet membranes were incubated with 6.4 nM [^{3}H]epinephrine for 2 hr at 15°. Dissociation was initiated by addition of 10 μM phentolamine simultaneously with 0.1 mM Gpp(NH)p or 120 mM NaCl, or both, in a volume which diluted the incubation 10%. Dissociation was monitored at the time points shown by transferring 1.0-ml aliquots of a bulk incubation to 5 ml of ice-cold Buffer A, and vacuum filtration through Whatman GF/C filters, as described under Methods. The specific binding of [^{3}H]epinephrine at time zero (just prior to initiating dissociation) was 1240 cpm/1-ml aliquot, which was equivalent to 61 fmoles/mg of membrane protein. This experiment is representative of five separate experiments performed in duplicate or triplicate.

B. Dissociation of [^{3}H]epinephrine from prelabeled agonist-*alpha*-receptor complexes subsequent to solubilization. Human platelet membranes were prelabeled with 10.6 nM [^{3}H]epinephrine for 2 hr at 15° prior to solubilization, as described under Methods. Dissociation, studied at 15°, was initiated by the addition of 120 mM NaCl or 0.1 mM Gpp(NH)p, or both, using 120 mM *N*-methyl-D-glucamine to maintain a constant ionic strength in incubations that did not include NaCl. At the time points shown, the amount of radioligand remaining bound to 0.5-ml aliquots of the solubilized preparation was determined by using Sephadex G-50 chromatography. The results are from one experiment representative of three separate experiments performed in triplicate. The binding of [^{3}H]epinephrine at time zero was 2104 cpm/0.5-ml aliquot in the experiment shown, which is equivalent to 12 fmoles/mg of solubilized membrane protein.

DISCUSSION

Sodium ion has been implicated as an important modulator of receptors coupled to inhibition of adenylate cyclase activity. The present studies demonstrate that sodium ion affects both *alpha*-receptor-agonist as well as *alpha*-receptor-antagonist interactions at human platelet *alpha*-adrenergic receptors coupled to inhibition of basal and prostaglandin E_1-stimulated adenylate cyclase. However, it should be noted that the effects of monovalent cations on *alpha*-receptor-agonist interactions are phenomenologically opposite to effects on *alpha*-receptor-antagonist interactions. Thus, whereas sodium decreases receptor affinity for agonist agents, sodium appears to increase slightly the receptor affinity for antagonist agents as well as to increase slightly the apparent density of *alpha*-adrenergic receptor sites. The physiological significance, if any, of the small effects of Na^+ on receptor-antagonist interactions remains to be determined. Nonetheless, the similar specificity ($Na^+ > Li^+ > K^+$) and potency (EC_{50} for Na^+ approximately 5–15 mM) of monovalent cations in modulating the affinity of *alpha*-receptor-agonist and *alpha*-receptor-antagonist interactions suggest that the same binding site may mediate both effects of Na^+. However, the K^+ ion is considerably more potent in modifying receptor-antagonist than receptor-agonist interactions (Fig. 3 and Fig. 5).

Although both Na^+ and guanine nucleotides have been implicated as having important roles in hormonal inhibition of adenylate cyclase (1) and both modulate *alpha*-adrenergic receptor-agonist interactions in platelet membrane preparations, certain findings distinguish the effects of Na^+ from those of guanine nucleotides on the platelet *alpha*-adrenergic system. First, as demonstrated in the present studies, Na^+ modifies both *alpha*-receptor-agonist and *alpha*-receptor-antagonist interactions in human platelet membranes. In contrast, guanine nucleotides have not been reported to alter *alpha*-receptor-antagonist interactions in the human platelet (10, 11). Second, although both Na^+ and guanine nucleotides decrease the affinity of $alpha_2$-receptors for agonists, the characteristics of *alpha*-receptor-agonist interactions have been reported to be qualitatively different in the presence of 120 mM Na^+ versus 0.1 mM Gpp(NH)p. Thus Gpp(NH)p both decreases agonist potency and removes the apparent heterogeneity of receptor-agonist interactions observed in the absence of exogenous nucleotide effectors and manifested by a shallow (i.e., pseudo-Hill coefficient <1) competition binding curve (9–11). In contrast, a quantitative comparison of the shapes of agonist competition curves using computer modeling techniques has indicated that Na^+ does not appear to abolish the heterogeneity of *alpha*-receptor-agonist interactions in rabbit platelet membranes (13), although such a discrepancy in the shapes of the competition profiles in the presence of Gpp(NH)p versus Na^+ was not noted in the present studies on human platelet membranes (cf. Fig. 1). Finally, the present studies demonstrate that the sensitivity of *alpha*-receptor-ligand interactions to sodium ion is retained following digitonin solubilization, regardless of whether the *alpha*-adrenergic receptor population is solubilized from control (unoccupied) membranes (Fig. 4) or from membranes prelabeled with the agonist, [^{3}H]epinephrine (Fig. 6), or the antagonist, [^{3}H]yohimbine (Fig. 5). This finding is in distinct contrast to the sensitivity of the detergent-solubilized *alpha*-adrenergic receptor to guanine nucleotides. Thus, the ability of guanine nucleotides to decrease *alpha*-receptor affinity for agonists is not observed following solubilization unless the *alpha*-receptors are first occupied by the agonist [^{3}H]epinephrine prior to exposure to digitonin (9, 10). This retention of guanine nucleotide sensitivity of the prelabeled [^{3}H]epinephrine-*alpha*-receptor complex is paralleled by an increase in apparent size of the *alpha*-receptor (9, 10, 14), suggesting that agonist occupancy of the human platelet *alpha*-receptor promotes or stabilizes receptor interaction with the GTP-binding protein modulating *alpha*-receptor affinity for agonists. The present observations that the sensitivity of solubilized *alpha*-adrenergic receptors to sodium is retained regardless of whether or not the receptors are occupied at the time of solubilization provide evidence that the membrane component conferring sensitivity of the *alpha*-adrenergic receptor to guanine nucleotides is distinct from that conferring sensitivity to sodium ion. These data thus argue against the hypothesis of Aktories *et al.* (15) that the site conferring sensitivity to Na^+ might be the GTP-binding protein. It is, in fact, possible that the receptor moiety itself possesses the Na^+ binding site responsible for modulating *alpha*-receptor-ligand interactions, since sensitivity to sodium is demonstrated by unoccupied, antagonist-occupied, or agonist-occupied solubilized $alpha_2$-receptors.

The mechanism by which sodium ion enhances hormonal inhibition of adenylate cyclase is not yet understood (1, 16, 17). However, the ability of sodium to decrease the potency of (−)-epinephrine in attenuating human platelet catalytic activity (18) is consistent with the effects of sodium on receptor-agonist interactions in the present study. The retention of sensitivity to sodium in digitonin-solubilized preparations reported in the present studies suggests a convenient way to isolate the sodium effector component by virtue of its association with the *alpha*-adrenergic receptor. It is hoped that future studies will elucidate whether or not the effects of Na^+ are mediated by a macromolecule distinct from the receptor moiety and, furthermore, clarify the functional relevance of this component in mediating *alpha*-adrenergic physiological effects.

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