

Parallel inactivation of α_2 -adrenergic agonist binding and N_i by alkaline treatment

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α_2 -Adrenergic receptor-mediated inhibition of adenylate cyclase requires the guanine nucleotide-binding protein, N_i . This protein may also be required for stabilization of high-affinity α_2 -adrenergic agonist binding. Human platelet membranes treated under alkaline conditions (pH 11.5) exhibited a selective loss of high-affinity agonist binding as measured by *p*-[³H]aminoclonidine and [³H]UK 14,304. Binding of the antagonist [³H]yohimbine was largely unaffected with retention of >60% of control binding sites. N_i , determined by pertussis toxin-catalyzed [³²P]ADP-ribosylation of cholate extracts from alkaline-treated membranes, was also markedly reduced. The parallel loss of α_2 -agonist binding and N_i provides additional evidence that N_i is required for α_2 -adrenergic agonist binding.

α_2 -Adrenergic receptor Agonist binding Nucleotide-binding protein Alkaline treatment

1. INTRODUCTION

Adrenergic regulation of adenylate cyclase can be either stimulatory or inhibitory. Agonist-induced attenuation of adenylate cyclase activity has been demonstrated in several systems including those containing α_2 -adrenergic [1], muscarinic [2] and opiate receptors [3]. This effect requires a guanyl nucleotide regulatory protein, N_i [4]. Agonist binding to inhibitory receptors may also involve an interaction with N_i , but this is less well characterized. GTP analogs decrease the affinity of α_2 -adrenergic agonist binding with no decrease in antagonist binding [5,6] or even an increase [7].

Abbreviations: IAP, islet-activating protein (pertussis toxin); N_i , inhibitory guanine nucleotide-binding protein; N_s , stimulatory guanine nucleotide-binding protein; PAC, *p*-aminoclonidine; *P/Y*, ratio of specifically bound 3 nM [³H]PAC/10 nM [³H]yohimbine; TM, buffer containing 50 mM Tris-Cl, pH 7.6, 10 mM MgCl₂; TME, TM buffer supplemented with 1 mM EGTA; UK 14,304, 5-bromo-6-*N*-[2-(4,5-dihydroimidazolyl)]quinoxaline

A similar reduction of agonist binding has also been demonstrated for membranes from cells treated with pertussis toxin [8–10]. Because of these observations, it has been hypothesized that inhibitory agonist binding to human platelet α_2 -receptors requires N_i .

Citri and Schramm reported a selective inactivation of the stimulatory guanine nucleotide regulatory protein, N_s , and the catalytic subunit of adenylate cyclase, *C*, after treatment of turkey erythrocyte membranes with alkaline buffer [11, 12]. Here, we report a selective abolition of high-affinity α_2 -agonist binding without significant inactivation of antagonist binding following pretreatment of human platelet membranes under alkaline conditions. This effect is correlated with a parallel decrease in pertussis toxin-catalyzed [³²P]ADP-ribosylation of N_i . These observations provide additional evidence that high-affinity agonist binding to platelet α_2 -adrenergic receptors requires a functional N_i protein and provides a new tool for studying this interaction. A preliminary report of this work has been presented in abstract form [13].

2. MATERIALS AND METHODS

2.1. Purification of platelet membranes

Purified human platelet membranes were prepared by the method of Neubig and Szamraj [15] and stored at -70°C for up to 6 weeks prior to use.

2.2. Alkaline treatment

Platelet membranes were thawed, homogenized with 5 strokes in a Potter-Elvehjem homogenizer, and diluted 20-fold with 50 mM sodium phosphate (pH 7.6–12.0). After incubation on ice for 1 h, membranes were collected by centrifugation at $145\,000 \times g$ for 30 min and resuspended in buffer A (50 mM Tris-Cl, 10 mM MgCl_2 , 1 mM EGTA, pH 7.6) for binding assays and pertussis toxin labelling.

2.3. Binding assays and pertussis toxin labelling

Reagents and methods for measurement of [^3H]-yohimbine, *p*-[^3H]aminoclonidine and [^3H]UK 14,304 binding, cholate extractions and pertussis labelling of N_i were as described [14]. Binding data were determined in triplicate and are presented as mean \pm SD. Curvilinear Scatchard plots were analyzed using the SCAFIT computer program [16].

3. RESULTS

Incubation of purified platelet membranes at a pH up to 11.0 for 1 h did not affect binding of 10 nM [^3H]yohimbine; however, at pH 11.5–12, there was a partial reduction in binding (fig.1). High-affinity agonist binding as measured by 3 nM [^3H]PAC was also unaffected to pH 11.0, but was completely lost at pH 11.5 or greater (fig.1). This effect of pH was complete in less than 30 min (not shown); consequently, 1 h was used as a standard incubation condition.

Equilibrium binding curves for [^3H]PAC and [^3H]yohimbine were performed with membranes pretreated in 50 mM phosphate buffer, pH 7.6 (control) and pH 11.5 (pH 11.5 membranes). There was no significant change in K_d and only a slight reduction in B_{max} for yohimbine binding to pH 11.5 membranes (fig.2A). Analysis using SCAFIT showed the best fit to a one-site model with a K_d and B_{max} for control membranes of

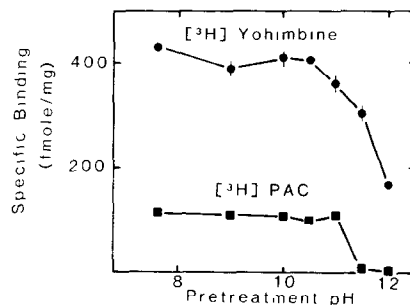


Fig.1. Dependence of binding to [^3H]PAC and [^3H]yohimbine on pretreatment pH. Equilibrium binding of 3 nM [^3H]PAC (■) and 10 nM [^3H]yohimbine (●) was measured for membranes pretreated with 50 mM sodium phosphate at the indicated pH and returned to pH 7.6 as described in section 2. Data are presented as mean \pm SD of triplicate determinations of specific binding per mg measured protein. The experiment shown is representative of 4 such experiments with similar results.

3.9 ± 0.5 nM and 400 ± 115 fmol/mg ($n=4$). Because of the removal of approx. 50% of the protein, the actual recovery of yohimbine binding sites was 50–60% following alkaline pretreatment.

The PAC binding curve for control membranes exhibits a nonlinear Scatchard plot (fig.2B). A 2-site fit was appropriate with $K_{d1}=0.7$ nM, $K_{d2}=8$ nM, $B_1=76$, $B_2=198$ fmol/mg [14]. In contrast, pH 11.5-treated membranes are characterized by only a very small amount of [^3H]PAC binding (fig.2B). The high-affinity [^3H]PAC binding seen for control membranes is not present with the pH 11.5 membranes. Binding is best fitted by a 1-site model with K_d , 3.4 ± 0.1 nM and B_{max} , 100 ± 20 fmol/mg ($n=3$).

To assess the effect of alkaline treatment on the inhibitory guanine nucleotide-binding protein, pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of the α -subunit (M_r 41 000) of N_i was used to quantitate amounts of that protein in cholate extracts from membranes pretreated at pH ranging from 7.6 to 12.0 (fig.3). There is a gradual decrease of approx. 50% in the amount of labelling of the N_i α -subunit from pH 7.6 to 11.0. At pH 11.5 a nearly complete abolition of labelling occurs. This sharp decrease parallels the decrease observed in high-affinity agonist binding. In some preparations of pH-pretreated membranes, we observed an increase in labelling at pH 10.0; however, in all cases, there was a decrease for pH 11.0 or greater.

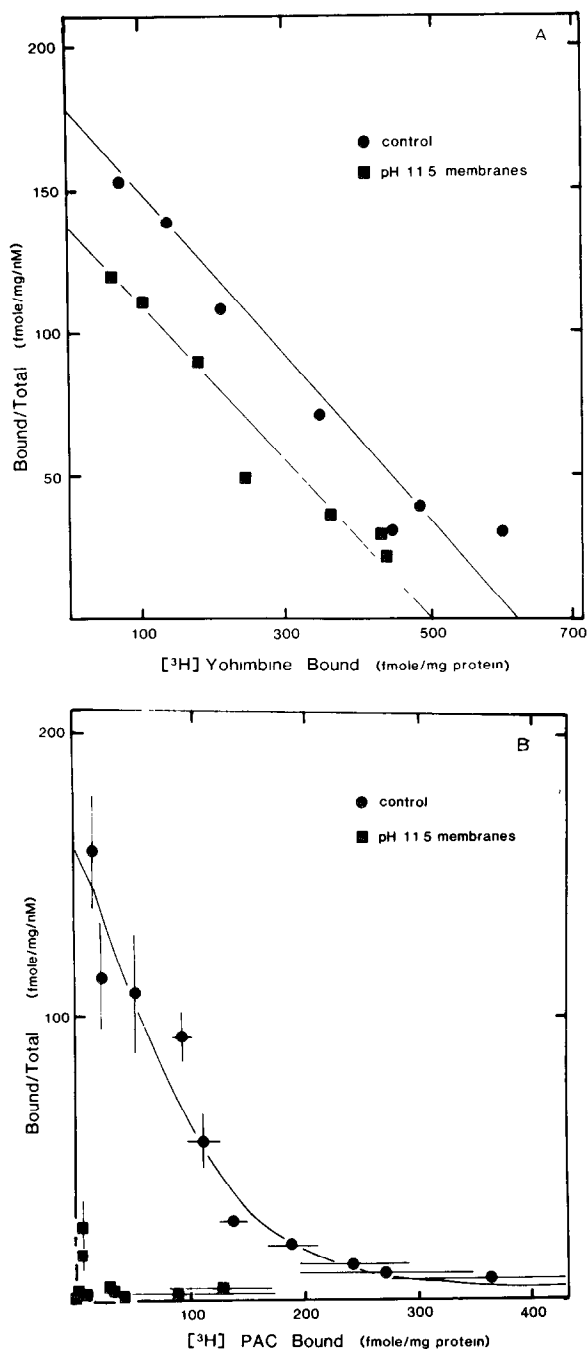


Fig.2. Scatchard transformation of the binding of (A) $[^3\text{H}]$ yohimbine and (B) $[^3\text{H}]$ PAC to control (●) and pH 11.5-pretreated (■) membranes. Binding was measured as described in section 2. Data are expressed as fmol/mg measured protein and are from a single experiment representative of (A) 6 and (B) 3 experiments with similar results.

41 K —

pH 7.6 9.0 10.0 10.5 11.0 11.5 12.0

Fig.3. $[^{32}\text{P}]\text{NAD}$ labelling of N_i in cholate extracts prepared from membranes pre-treated at various pH values. IAP-catalyzed N_i labelling by $[^{32}\text{P}]\text{NAD}$ was performed as in section 2 followed by SDS-polyacrylamide gel electrophoresis. The dried gel was exposed to X-ray film for 6 h and a single labelled band was observed with $M_r = 40\,700$ [13]. No radioactivity was observed in the lane without added IAP. Three other experiments were performed with similar results.

A comparison of the amount of ^{32}P incorporated into the 41 kDa protein with a measure of high-affinity agonist binding (P/Y , see figure legend) is plotted as a function of pH (fig.4). There is a marked decrease in high-affinity binding with a concomitant abolition of N_i labelling at pH 11.5 or greater. Interestingly, inactivation of $\sim 50\%$ of N_i (pH 10.5) occurs without any significant effect on agonist binding. [Note: Recent experiments with pertussis toxin treatment of platelet mem-

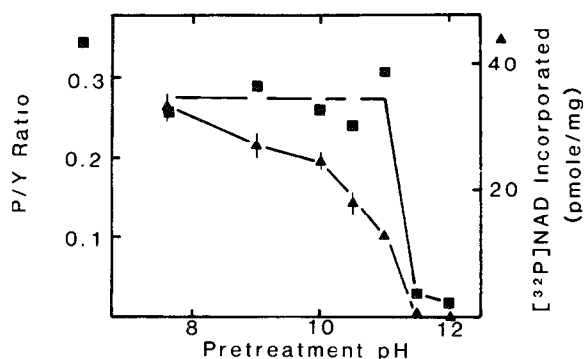


Fig.4. Dependence of P/Y and $[^{32}\text{P}]\text{NAD}$ incorporation on alkaline pH pre-treatment. Data from fig.1 were used to calculate P/Y , the ratio of 3 nM $[^3\text{H}]\text{PAC}/10$ nM $[^3\text{H}]\text{yohimbine}$ specifically bound. This index of high-affinity agonist binding is plotted as a function of pretreatment pH (■). Errors of triplicates are $<20\%$. N_i labelling of 2% cholate extracts (▲) was quantitated by excising and counting the labelled bands from the gel in fig.4. Error bars depict the ranges of duplicate lanes. Background counts from the lane containing no pertussis toxin (150 cpm) were subtracted from all points. Data are presented in pmol NAD per mg starting protein ($5\ \mu\text{g}$ per point).

branes show that modification of 50–70% of the N_i is without effect on [3H]PAC binding. Complete modification of N_i in membranes has not yet been achieved (Kim and Neubig, unpublished.) The amount of pertussis toxin substrate (N_i) in controls (pH 7.6) was 28 (range 9–47) pmol [^{32}P]NAD incorporated/mg protein ($n=4$) compared with that for pH 11.5-treated membranes, which was 3.0 (range 1–6) pmol/mg protein ($n=3$). The binding of the full α_2 -adrenergic agonist [3H]UK 14,304 is also reduced by alkaline treatment. The ratio of specific binding of 2 nM [3H]UK 14,304 to that of 10 nM [3H]yohimbine was reduced from the control value of pretreated membranes 0.39 ± 0.04 , to 0.05 ± 0.01 in pH 11.5 ($n=3$).

4. DISCUSSION

We show here that treatment of purified platelet membranes under extreme alkaline conditions (pH 11.5) results in the selective inactivation of binding of the partial α_2 -agonist, [3H]PAC, and full α_2 -agonist [3H]UK, 14,304 but not that of the antagonist, [3H]yohimbine. This is the first report of the effect of alkaline treatment on adrenergic agonist binding. Because the results obtained following pH 11.5 treatment resembled those seen for PAC binding in the presence of 10^{-5} M GppNHP [14], we hypothesized that the selective reduction of agonist binding might be due to loss of N_i . Indeed, N_i measured by pertussis toxin catalyzed [^{32}P]ADP-ribosylation is inactivated by alkaline treatment (fig.4). These observations parallel those of Citri and Schramm [11,12] on alkaline treatment of β -adrenergic receptors where N_s is inactivated but antagonist binding is preserved.

Selective reduction of α_2 -agonist binding has also been reported for the reversible reagents sodium [17] and GppNHP [14] and irreversible reagents *N*-ethylmaleimide [18,19], chymotrypsin [20], heat [19] and pertussis toxin [8–10]. Only for the latter is the mechanism known to involve effects on the inhibitory guanine nucleotide-binding protein, N_i . The parallel loss of pertussis toxin substrate and α_2 -agonist binding between pH 11.0 and 11.5 suggests that inactivation of N_i results in the selective loss of high-affinity agonist binding. Final proof of this mechanism would require

reconstitution of high-affinity agonist binding with purified N_i . This type of experiment has recently been performed using solubilized muscarinic receptors [21].

Although the complete inactivation of N_i parallels the loss of [3H]PAC binding, in each experiment there was some decrease in pertussis toxin substrate at pH 10.5–11 without loss of agonist binding. It is possible that alkaline treatment affects the activity of N_i as a pertussis toxin substrate (e.g. by dissociating the subunits) without affecting its ability to couple to receptors. Another likely explanation can be found in the fact that there is a 25–100-fold excess of N_i protein over α_2 -receptors in platelet membranes [14]. Consequently, inactivation of 50–80% of the N_i would still leave more than enough to couple to α_2 -adrenergic receptors² (see *Note*, p. 323).

Recently, Childers and co-workers [22,23] studied the effects of acid (pH 4.5) treatment of brain membranes on opiate receptors. Control agonist binding was apparently not changed but the sensitivity to guanine nucleotides was increased. Also, opiate receptor-mediated inhibition of adenylate cyclase was increased. The relation of these observations to our present findings is not clear. Effects of alkaline treatment on receptor-mediated inhibition of adenylate cyclase in our system cannot be determined because catalytic activity is completely inactivated at pH 11 (not shown).

Our data provide additional evidence for the role of the guanine nucleotide-binding protein, N_i , in promoting high-affinity α_2 -agonist (and partial agonist) binding. Alkaline treatment should prove a useful tool in studying receptor- N_i interactions.

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