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IDENTIFICATION AND REGULATION OF ALPHA 2-ADRENERGIC RECEPTORS IN RABBIT ILEAL MUCOSA

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Abstract—The alpha 2-adrenergic receptors in rabbit ileal mucosal membranes can be identified by using [^{3}H]clonidine. [^{3}H]Clonidine bound to a homogeneous population of sites (30–120 fmoles/mg protein) with a K_D of 2.2 nM at 25°. Alpha-adrenergic agonists and antagonists competed with [^{3}H]clonidine for the binding sites with an order of potency typical for alpha 2-receptors. Mg^{2+}, Ca^{2+}, or Mn^{2+} (2.4 mM) markedly increased the binding of [^{3}H]clonidine. At the maximally effective concentration, Mg^{2+} increased both the binding affinity of [^{3}H]clonidine and the number of receptor sites. Both NaCl and GppNHp, the guanyl nucleotide, inhibited [^{3}H]clonidine binding. NaCl decreased the binding affinity of [^{3}H]clonidine as well as the number of receptor sites. In contrast, GppNHp decreased only the binding affinity of [^{3}H]clonidine, with no appreciable effect on the number of receptor sites. These findings indicate that ileal mucosal alpha 2-receptors can exist in multiple affinity states, which can be regulated by divalent cations, NaCl, and guanyl nucleotides. It appears that NaCl and GppNHp regulate alpha 2-receptors in ileal mucosa by different mechanisms.

The catecholamines epinephrine and norepinephrine have been shown to enhance NaCl absorption and to decrease HCO_3^- secretion in isolated ileal mucosa [1]. The effects on ion transport in the small intestine are regarded as alpha-adrenergic responses because the alpha-adrenergic antagonist phentolamine, but not the beta-adrenergic antagonist propranolol, can block the effects of epinephrine and norepinephrine [1, 2]. Further studies with yohimbine, an antagonist specific for alpha 2-adrenergic receptors, have demonstrated that the alpha-adrenergic receptors which regulate ion transport in the ileal mucosa can be classified as the alpha 2-receptor subtype [3]. Recently, alpha 2-adrenergic receptors in epithelial cells of ileal mucosa have been identified by using [^{3}H]yohimbine and [^{3}H]*p*-aminoclonidine, a partial agonist [4, 5]. In the present study, [^{3}H]clonidine, a potent agonist in ileal mucosa [3], was used to identify alpha 2-receptors. In addition, the effects of NaCl, several divalent cations, and a guanyl nucleotide, known regulators of alpha 2-receptors in other tissues [6–9], have been examined in ileal mucosa. The effects of these ions are of particular interest because the alpha 2-adrenergic intestinal antisecretory response is associated with ion transport across the mucosa [1].

MATERIALS AND METHODS

[^{3}H]Clonidine (23.8 Ci/mmole) was obtained from New England Nuclear (Boston, MA). (−)Epinephrine bitartrate, (−)norepinephrine bitartrate, yohimbine HCl, (±)isoproterenol HCl, serotonin, and 5′-guanylyl imidodiphosphate (GppNHp) were purchased from the Sigma Chemical Co. (St. Louis, MO); (+)epinephrine bitartrate, (+)norepinephrine bitartrate, and oxymetazoline were from Sterling-Winthrop Research Institute (Rensselaer, NY). Clonidine HCl was from Ingelheim-Boehringer (Ridgefield, CT), prazosin from Pfizer Inc. (New York, NY), methoxamine HCl from the Burroughs Wellcome Co. (Research Triangle Park, NC), guanabenz acetate from Wyeth Laboratories (Philadelphia, PA), and (−)propranolol HCl from Ayerst Laboratories (New York, NY).

Preparation of ileal mucosal membranes. Male New Zealand white rabbits (2–3 kg), fed with a standard rabbit chow and water *ad lib.* were killed by an overdose of pentobarbital administered via ear vein. The distal ileum was removed quickly, flushed with cold saline solution, and opened longitudinally. The mucosa was removed by gentle scraping with a microscope slide. Histological examination showed that the majority of tissue removed by this method consisted of mucosal cells.

Mucosal membranes were prepared using two kinds of Tris buffer, depending on the nature of the experiment. Tris buffer solution (50 mM, pH 7.5) containing 5 mM $MgCl_2$ was used for the identification of alpha 2-adrenergic receptors. Tris buffer solution (50 mM, pH 7.5) containing 1 mM EDTA was used to prepare membranes for the experiments on the effects of Mg^{2+}, Na^+ and guanyl nucleotide on [^{3}H]clonidine binding.

All procedures for the preparation of membranes were carried out at 4°. The mucosa was homogenized in Tris buffer solution for three 10-sec periods using a Brinkmann Polytron at a setting of 6. The homogenates were centrifuged at 1100 *g* for 10 min, and the resulting supernatant fraction was filtered through three layers of cheesecloth and then centrifuged at 30,000 *g* for 10 min. The pellet was washed twice by homogenization and centrifugation in Tris buffer solution. The final pellet was rehomogenized

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in Tris buffer solution with a Teflon–glass homogenizer. The protein concentration of the final membrane suspension was 5–8 mg/ml.

Binding assay. The receptor binding studies were carried out in a final volume of 0.16 ml of incubation medium. An incubation medium consisting of 50 mM Tris–HCl/5 mM $MgCl_2$ (pH 7.5) was used for identification of alpha-2 receptors. An incubation medium consisting of 50 mM Tris–HCl and 1 mM EDTA with indicated ions or guanyl nucleotide added was used for examination of the effect of Mg^{2+}, Na^+ and guanyl nucleotide on [^{3}H]clonidine binding. The membrane protein in the binding assay was about 0.5 to 0.9 mg. The incubation was initiated by the addition of [^{3}H]clonidine and continued for 15 min at 25°. Incubation was terminated by adding 5 ml of ice-cold incubation medium, followed by rapid filtration of the mixture under vacuum through Whatman GF/B glass fiber filters. The filters were further washed with 10 ml of incubation medium at 4°. This wash reduced nonspecific binding without decreasing specific binding. The filters were placed in a Triton-based solution and counted by liquid scintillation spectrometry at an efficiency of 39%. The specific binding of [^{3}H]clonidine was defined as the difference between counts in the absence and presence of 0.01 mM (−)epinephrine. The rationale for using 0.01 mM (−)epinephrine to define nonspecific binding is described below. For routine assay, the concentration of [^{3}H]clonidine in the incubation medium was 2 nM, and the specific binding ranged from 40 to 70% of the total binding. Binding data described under results refer to specific binding unless otherwise indicated. Protein was determined by the method of Lowry *et al.* [10].

Definition of nonspecific binding. (−)Epinephrine was found to inhibit total binding of [^{3}H]clonidine in a biphasic manner (Fig. 1). The first phase of inhibition reached a plateau around 1 μM. No further inhibition was observed when the concentration of (−)epinephrine was increased to 0.1 mM. The second phase of inhibition became apparent when the concentration of (−)epinephrine was increased to 1 mM. These findings suggested that [^{3}H]clonidine bound to both high and low affinity binding sites with the affinity differing by more than 100 times. Experiments using (−)norepinephrine to inhibit the total binding of [^{3}H]clonidine gave similar biphasic responses with the same inhibition plateau as (−)epinephrine in the first phase of inhibition. Antagonists phentolamine and yohimbine at higher concentrations ($>10^{-6}$ M) also displaced [^{3}H]clonidine from the low affinity binding sites. The binding of [^{3}H]clonidine to low affinity sites was determined to be nonspecific because weak alpha 2-adrenergic agents such as phenylephrine and (±)isoproterenol were more effective than (−)epinephrine in interacting with the low affinity sites (Fig. 1). Therefore, [^{3}H]clonidine binding which was not displaced by (−)epinephrine at 0.01 mM was considered to be nonspecific binding.

RESULTS

Identification of alpha 2-adrenergic receptors with [^{3}H]clonidine. Binding of [^{3}H]clonidine (2 nM) to rabbit ileal mucosal membranes reached equilibrium in less than 10 min at 25° and was reversible. The binding of [^{3}H]clonidine was saturable (Fig. 2), and a Scatchard plot [11] showed that [^{3}H]clonidine bound to a homogeneous population of binding sites (Fig. 2 inset). The maximum number of binding sites varied from 30 to 120 fmoles/mg protein in different membrane preparations, and the dissociation con-

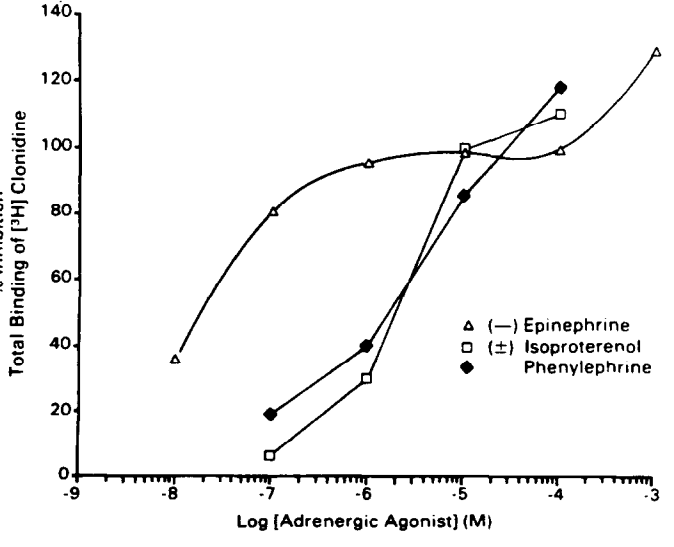

Fig. 1. Inhibition of total binding of [^{3}H]clonidine by adrenergic agonists. Mucosal membranes were incubated with [^{3}H]clonidine (2 nM) in the absence and presence of various concentrations of adrenergic agonists, and total binding was determined. The incubation buffer solution consisted of 50 mM Tris–HCl and 5 mM $MgCl_2$, pH 7.5. Inhibition of total binding by 0.01 mM (−)epinephrine is given the value of 100% inhibition. Each value is the mean of triplicate determinations. The results are representative of two such experiments.

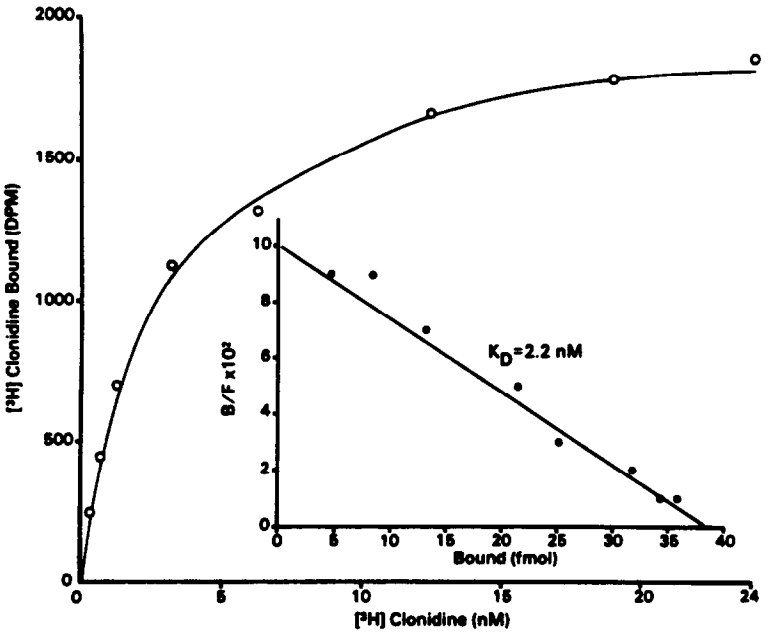

Fig. 2. Specific binding of [³H]clonidine to rabbit ileal mucosal membranes as a function of increasing concentration of [³H]clonidine. Mucosal membranes (0.75 mg protein) were incubated with various concentrations of [³H]clonidine, and the specific binding was determined. The incubation buffer solution consisted of 50 mM Tris–HCl and 5 mM $MgCl_2$, pH 7.5. Each value is the mean of triplicate determinations. The figure shown is representative of three such experiments. The inset shows Scatchard analysis of [³H]clonidine binding to mucosal membranes. B/F is the ratio of bound [³H]clonidine to free [³H]clonidine. The slope, which was determined by linear regression analysis, is equal to $-1/K_D$, where K_D is the dissociation constant of [³H]clonidine. The number of binding sites is calculated from the intercept of the plot with the abscissa.

stant of [³H]clonidine was 2.2 ± 0.2 nM (N = 3). Table 1 shows the concentrations of agents required to induce a 50% inhibition of [³H]clonidine binding. The order of potency of adrenergic agonists in competing with [³H]clonidine for the binding sites was clonidine > (−)epinephrine > (−)norepinephrine > (±)isoproterenol. These findings are consistent with the classical pattern of alpha-adrenergic responses induced by these agents in ileal mucosa [1, 3]. The [³H]clonidine binding sites showed stereospecificity with (−)catecholamine isomers being much more potent than their corresponding (+)isomers in competing with [³H]clonidine for the binding sites. The alpha-adrenergic antagonists phentolamine and WB 4101 were potent inhibitors of [³H]clonidine binding, whereas dopamine, serotonin, and the beta-adrenergic blocker propranolol were very weak inhibitors of [³H]clonidine binding ($IC_{50} \geqslant 10^{-6}$ M). Oxymetazoline, guanabenz, naphazoline, and tetrahydrazoline, which are known potent intestinal alpha 2-adrenergic agonists [12, 13], were also potent inhibitors of [³H]clonidine binding. Lidamidine, which has been reported recently to have a weak effect on ion transport in isolated rabbit ileal mucosa [14], was also a weak inhibitor of [³H]clonidine binding. These data indicated that [³H]clonidine binding sites have characteristics expected of alpha 2-adrenergic receptors. Furthermore, yohimbine, which is an alpha 2-adrenergic antagonist, was at least several hundred times more potent than prazosin, a specific alpha 1-adrenergic antagonist, as an inhibitor of [³H]clonidine binding. Phenylephrine and methoxamine, which are potent alpha 1-adrenergic agonists, were only weakly active at [³H]clonidine binding sites (Table 1). These results indicate that [³H]-clonidine binding sites can be classified as alpha 2-receptor sites. The slopes (pseudo Hill coefficients) of the competition curves of clonidine, (−)epinephrine, phentolamine, and yohimbine are approximately equal to 1 (Table 1), indicating that all these adrenergic agents bound to a homogeneous population of binding sites.

Effects of Mg^{2+}, Ca^{2+} *and* Mn^{2+} *on [³H]clonidine binding.* When Mg^{2+} was not added to the incubation medium, little specific binding of [³H]clonidine was observed. Mg^{2+} at 2.4 mM markedly increased (by 5-fold) the specific binding of [³H]clonidine (2 nM) without any effect on nonspecific binding. The effect of Mg^{2+} was found to be dose dependent (Fig. 3). Mg^{2+} at 0.01 mM markedly enhanced [³H]clonidine binding. The half-maximal and maximal effects of Mg^{2+} occurred at 0.1 and 2.4 mM respectively. The binding of [³H]clonidine was also greatly enhanced by Ca^{2+} and Mn^{2+}, with a maximal effect similar to that of Mg^{2+} (Fig. 3). When the maximally effective concentration of Ca^{2+} or Mn^{2+} was added to Mg^{2+}, neither of these ions enhanced the increase in specific binding of [³H]clonidine beyond that induced by Mg^{2+} alone. A Scatchard plot analysis (Fig. 4) indicates that Mg^{2+} at 2.4 mM increased the number of receptor sites by 85 ± 9% (N = 3) and increased the affinity of [³H]clonidine for the receptor sites by 2.7-fold (the dissociation constant of [³H]clonidine decreased from 6.6 ± 1.9 to 1.8 ± 0.4 nM, N = 3).

Table 1. Inhibition of [^{3}H]clonidine binding by adrenergic, and other, agents*

Compound	IC_{50} (nM)	Pseudo Hill coefficients
Alpha-agonists		
Clonidine	4.6 ± 1	1.1
Oxymetazoline	18 ± 5	
Guanabenz	16 ± 0.5	
Naphazoline	4.8 ± 1.1	
Tetrahydrazoline	17 ± 0.5	
(−)Epinephrine	15.6 ± 0.7	1.1
(+)Epinephrine	113 ± 27	
(−)Norepinephrine	47 ± 2.7	
(+)Norepinephrine	717 ± 159	
Alpha-methylnorepinephrine	60 ± 6	
Phenylephrine	400 ± 50	
Methoxamine	1800 ± 200	
Lidamidine	1450 ± 150	
Alpha-antagonists		
Phentolamine	32 ± 3	1.2
WB4101	90 ± 40	
Yohimbine	87 ± 15	0.95
Prazosin	>10,000	
Beta-agonist or antagonist		
(±)Isoproterenol	2000	
Propranolol	>10,000	
Others		
Dopamine	>10,000	
Serotonin	⩾10,000	

* IC_{50} is the concentration of each agent which caused 50% inhibition of [^{3}H]clonidine binding. The concentration of [^{3}H]clonidine in the binding assay was 2 nM. The IC_{50} of each agent was determined from the concentration-inhibition curve of [^{3}H]clonidine binding. The values shown are the means ± S.E.M. from two to four separate experiments. Pseudo Hill coefficients are from a representative experiment which was repeated once with similar results. Each value of the coefficients is not significantly different from one.

Effects of guanyl nucleotide and NaCl on [^{3}H] clonidine binding. Both NaCl and the guanyl nucleotide GppNHp, a GTP analog which is resistant to nucleotide phosphohydrolase action, inhibited [^{3}H] clonidine binding. The concentrations of NaCl and GppNHp required to produce a 50% inhibition of the binding of 2 nM [^{3}H]clonidine were 30 mM and 1 μM respectively (not shown). GppNHp at 100 μM and NaCl at ~150 mM each completely inhibited the binding of [^{3}H]clonidine. A Scatchard plot analysis showed that NaCl, at concentrations of 40 and 80 mM, decreased the number of receptor sites by 25 ± 5% (N = 2) and 50 ± 6% (N = 3) respectively. In addition, the affinity of [^{3}H]clonidine for the receptor sites was decreased by 1.2- (N = 2) and 1.5-fold (N = 3) by NaCl at 40 and 80 mM respectively (Fig. 5, data for 80 mM NaCl not shown). The effect of NaCl on [^{3}H]clonidine binding was due to Na^+ and not due to altered tonicity or Cl^- because sucrose and *N-methyl*-D-glucamine HCl were much weaker than NaCl in inhibiting [^{3}H]clonidine binding.

Although GppNHp at 1 and 5 μM decreased the affinity of [^{3}H]clonidine for the receptor sites by 1.4- (N = 2) and 1.9-fold (N = 3), respectively, it had no appreciable effect on the number of receptor sites (Fig. 6, data for 5 μM GppNHp not shown).

DISCUSSION

Recently [^{3}H]yohimbine and [^{3}H]*p*-aminoclonidine have been used to label alpha 2-receptors in epithelial cell membranes from small intestine [4, 5]. However, as Chang *et al.* [4] indicated, specific binding of these radioligands in epithelial cell membranes from rabbit ileum was low (15–25% of total binding). Similar low specific binding of [^{3}H]yohimbine has also been observed by us in rabbit ileal mucosal membranes. Although the specific binding of [^{3}H] yohimbine in epithelial cell membranes from the small intestine of rats was much higher (55% of total binding) [5], no data were presented in support of the definition concerning specific binding of [^{3}H] clonidine in that study. In this study, we used [^{3}H] clonidine to label alpha 2-receptors in rabbit ileal mucosal membranes. The results showed that the specific binding of [^{3}H]clonidine, as defined by inhibition by 0.01 mM (−)epinephrine, satisfied the criteria set forth for alpha 2-receptors: saturability, reversibility, stereospecificity, and appropriate specificity for alpha 2-receptor agonists and antagonists. This finding supports the use of the [^{3}H]clonidine binding technique for the investigation of alpha 2-receptors, which mediate the transport of ions, in

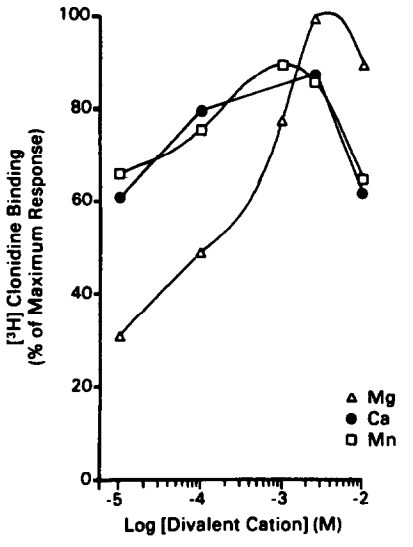

Fig. 3. Dose-dependent effects of magnesium chloride, calcium chloride and manganese chloride on the specific binding of [^{3}H]clonidine. Mucosal membranes were incubated with [^{3}H]clonidine (2 nM), and the specific binding was determined. The incubation medium consisted of 50 mM Tris–HCl and 1 mM EDTA, pH 7.5, with or without various concentrations (in excess of 1 mM EDTA) of divalent cations as indicated. Values for stimulation of specific binding of [^{3}H]clonidine by various cations are expressed as a percentage of the maximum increase in specific binding observed with 2.4 mM $MgCl_2$. Each value represents the mean of triplicate determinations from two separate experiments.

ileal mucosa. Since clonidine is a full alpha 2-agonist in ileal mucosa, the ability of [^{3}H]clonidine to label these alpha 2-receptors could make them a useful model for studying the clonidine–receptor interaction and for comparison with other alpha 2-receptors in platelets and renal cortex which produce only a partial agonist response to clonidine [7, 15].

Alpha 2-receptors in brain and platelets have been shown to exist in high and low affinity states for agonists, and are regulated by Na^+, divalent cations, and guanyl nucleotides [7–9, 16–20]. In the present study, Mg^{2+} markedly increased the binding of [^{3}H] clonidine binding sites as well as its binding affinity, which has also been shown for [^{3}H]epinephrine in human platelets [17]. Our results showed that Mn^{2+} and Ca^{2+} were potent stimulants as well for the binding of [^{3}H]clonidine and produced a maximum effect similar to Mg^{2+}. The fact that Mn^{2+} and Ca^{2+} did not further enhance the maximal effect of Mg^{2+} on [^{3}H]clonidine binding in ileal mucosa suggests that these ions may act on the same site(s) as Mg^{2+}. Both Mg^{2+} and Mn^{2+} have similar stimulatory effects on the binding affinity of agonists in platelets [6]. In contrast to platelets and ileal mucosa, Mn^{2+} stimulated the binding of [^{3}H]agonist to brain alpha 2-receptors, whereas Mg^{2+} had little [9] or much less effect than Mn^{2+} [20]. Furthermore, the effect of Mn^{2+} in the brain required the presence of guanyl nucleotide GTP. In the absence of GTP, the effect of Mn^{2+} is either abolished [9] or markedly reduced [20]. Evidence suggests that Mn^{2+} may have an action distinct from Mg^{2+} [9]. Despite these differences, however, the effect of Mn^{2+} on alpha 2-receptors in

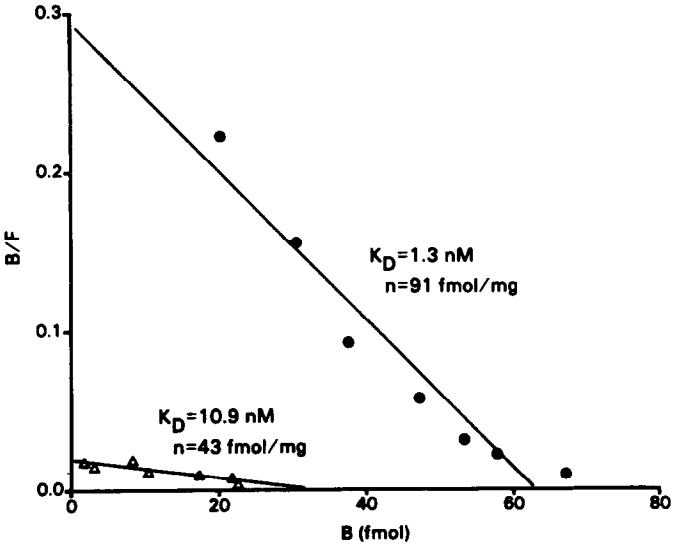

Fig. 4. Scatchard plot of [^{3}H]clonidine binding to mucosal membranes in the presence and absence of 2.4 mM $MgCl_2$. Mucosal membranes (0.68 mg protein) were incubated with various concentrations of [^{3}H]clonidine, and the specific binding was determined. The incubation medium consisted of 50 mM Tris–HCl and 1 mM EDTA, pH 7.5, with (●) or without (△) 2.4 mM $MgCl_2$ (in excess of 1 mM EDTA) as indicated. Each value is the mean of triplicate determinations from one experiment which had been repeated twice with similar results. K_D and number of binding sites, n, were determined as described in the legend of Fig. 2.

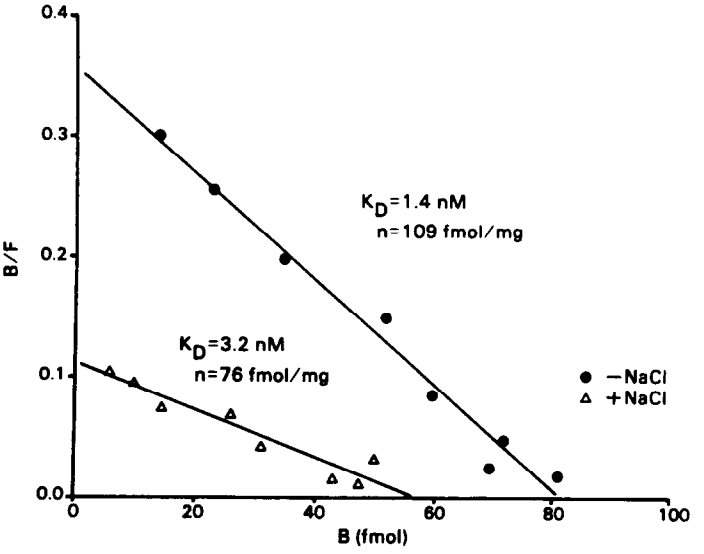

Fig. 5. Scatchard plot of [^{3}H]clonidine binding to mucosal membranes in the presence and absence of 40 mM NaCl. Mucosal membranes (0.74 mg protein) were incubated with various concentrations of [^{3}H] clonidine, and the specific binding was determined. The incubation medium consisted of 50 mM Tris–HCl, 1 mM EDTA, and 2.4 mM $MgCl_2$ (in excess of 1 mM EDTA), pH 7.5, with or without 40 mM NaCl as indicated. Each value is the mean of triplicate determinations from one experiment which was repeated once with similar results. K_D and the number of binding sites, n, were determined as described in the legend of Fig. 2.

the brain is comparable to the effect of Mg^{2+} on alpha 2-receptors in platelets and ileal mucosa in that it enhanced the binding of [^{3}H]agonist by increasing the number of high affinity receptor sites.

The mechanism(s) by which Mg^{2+} affects the number of receptor sites in ileal mucosa is unknown. Recent studies on human platelets and rat liver indicate that [^{3}H]agonists may selectively bind to the high affinity state of alpha 2-receptors, whereas [^{3}H] antagonists may bind to receptors existing in both high and low affinity states [16, 21]. [^{3}H]Clonidine was shown in this study to bind to a single class of binding sites which exhibited singular binding affinities for the agonists, (−)epinephrine and clonidine, and the antagonists, phentolamine and yohimbine, as indicated by the linear Scatchard plot and

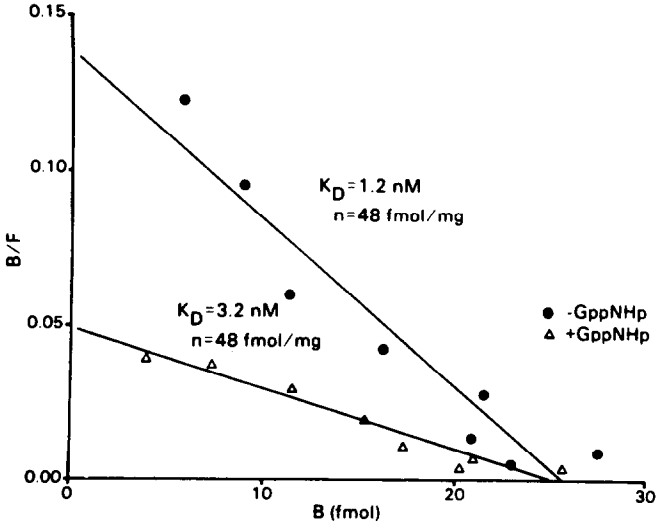

Fig. 6. Scatchard plot of [^{3}H]clonidine binding to mucosal membranes in the presence and absence of 1 μM GppNHp. Mucosal membranes (0.5 mg protein) were incubated with various concentrations of [^{3}H]clonidine, and the specific binding was determined. The incubation medium consisted of 50 mM Tris–HCl, 1 mM EDTA, and 2.4 mM $MgCl_2$ (in excess of 1 mM EDTA), pH 7.5, with or without 1 μM GppNHp as indicated. Each value is the mean of triplicate determinations from one experiment which was repeated once with similar results. K_D and the number of binding sites, n, were determined as described in the legend of Fig. 2.

the pseudo Hill coefficients being ~1. The increased number of [^{3}H]agonist binding sites in the presence of Mg^{2+} in human platelets is likely to be the result of the conversion of receptors from the low affinity state to the high affinity state [7, 16, 17, 21]. This could also be the case for the increased number of [^{3}H]clonidine binding sites due to Mg^{2+} in ileal mucosa. Alternatively, Mg^{2+} may have uncovered some latent receptors in ileal mucosa.

In contrast to Mg^{2+}, guanyl nucleotides and sodium have been reported to decrease the binding affinity of agonists, but not of antagonists, for alpha 2-receptors in several systems [4, 6, 7, 15, 21–24]. Our observation, that GppNHp reduced the binding affinity of [^{3}H]clonidine without changing the number of its binding sites, is comparable to the effect of GppNHp on alpha 2-receptors in human platelets, rat liver and rat renal cortex [4, 6, 7, 15, 21–24]. In these tissues, guanyl nucleotides favored the formation of the low affinity state of alpha 2-receptors. In contrast to GppNHp, NaCl had a dual effect on alpha 2-receptor sites in ileal mucosa; sodium decreased both the binding affinity of [^{3}H]clonidine and the number of its binding sites. Our findings suggest that sodium and guanyl nucleotide GppNHp reduced [^{3}H]clonidine binding via different mechanisms. In recent studies, Michel *et al.* [23] and Limbird and Speck [25] have also made the same conclusion by showing different regulatory effects of sodium and guanyl nucleotides on platelet alpha 2-receptors.

Since [^{3}H]agonists were reported to bind preferentially to the high affinity state of alpha 2-receptors in some peripheral tissues, further studies are needed on ileal mucosa to determine whether the effects of the divalent cations, Na^+ and GppNHp involved only the high affinity state of receptors or the entire population of receptors.

Although the physiological functions of these ions on alpha 2-receptors are unknown and remain to be elucidated, they are likely to play an important role in the regulation of alpha 2-receptor function in the small intestine since the activation of alpha 2-receptors in intestinal mucosa is indeed associated with a change in the permeability of the plasma membranes to ions such as sodium [1].

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