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Alpha$_2$-Adrenergic Receptor in Intestinal Epithelial Cells

Identification by [^{3}H]Yohimbine and Failure to Inhibit Cyclic AMP Accumulation

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SUMMARY

Alpha$_2$-adrenergic receptors in isolated rat intestinal epithelial cells were identified by using the *alpha*$_2$-selective antagonist [^{3}H]yohimbine. The contamination of *alpha*$_2$-adrenergic receptors in presynaptic nerve endings was ruled out by electron microscopic observations. The [^{3}H]yohimbine binding to the 100,000 × *g* pellet from the epithelial cells was saturable and of high affinity. Scatchard analysis yielded a K_D of 6.0 nM with a B_{max} of 37 fmoles of sites per milligram of protein. The binding was rapid and reversible. No cooperative interactions among the binding sites were observed. Inhibition of yohimbine binding by adrenergic agonists yielded the *alpha*$_2$-adrenergic potency series: clonidine > (±)-nordefrin > (−)-norepinephrine > (−)-epinephrine ≫ methoxamine > (−)-phenylephrine >>> (−)-isoproterenol. (−)-Isomers were more potent than (+)-isomers. The antagonist potency series also showed *alpha*$_2$-adrenergic specificity: yohimbine > dihydroergocryptine ≫ prazosin > phenoxybenzamine > propranolol. The inhibition potencies of [^{3}H]yohimbine binding by *alpha*-adrenergic agonists were correlated with those of the same agents for antidiarrheal effects *in vivo*. Clonidine (1 μM) failed to reduce the cyclic AMP levels augmented by prostaglandin E_1 (PGE_1) (30 μM) or vasoactive intestinal peptide in the presence of 3-isobutyl-1-methyl-xanthine in these isolated cells. Epinephrine (10 μM) in the absence or presence of pindolol (10 μM) did not reduce the PGE_1-augmented cyclic AMP levels. These results show that the intestinal epithelial cells contain *alpha*$_2$-adrenergic receptors through which *alpha*$_2$-adrenergic agonists may exert their antidiarrheal effect. It is suggested that the antidiarrheal effect of *alpha*$_2$-adrenergic agonists may not be due to the inhibition of adenylate cyclase in these cells.

INTRODUCTION

It has been well established that intestinal secretion plays a crucial role in the pathogenesis of diarrhea (1). The intestinal secretion is inhibited by epinephrine and norepinephrine (2–4). Moreover, the small intestinal epithelial cells are densely innervated by adrenergic neurons (5). These findings suggest significant roles of the endogenous catecholamines in regulating secretory diarrhea.

Recently we reported that the *alpha*$_2$-adrenergic agonists inhibit the intestinal secretion induced by cholera toxin (6), PGE_1,[2] and VIP (7). Intestinal secretagogues such as cholera toxin, PGE_1, and VIP induce intestinal secretion by elevating mucosal cyclic AMP levels (4). Although the stimulation of *alpha*$_2$-adrenergic receptors inhibits cyclic AMP accumulation in various intact cells or tissues (8–11), our previous results showed that the *alpha*$_2$ agonists clonidine and epinephrine failed to reduce the total mucosal cyclic AMP levels, but the drugs still inhibited the above secretagogue-induced intestinal secretion (6, 7). The measurement of total cyclic AMP levels in mucosa, however, was not a sensitive index of cyclic AMP levels within the epithelial cells, because the samples prepared from the whole mucosal layer contain non-epithelial cells. Since the intestinal epithelial cells play important roles in the intestinal secretion and are densely innervated by noradrenergic neurons, it seems reasonable to presume that the primary site of action of the *alpha*$_2$ agonists is the intestinal epithelial cells. Therefore, by using isolated intestinal epithelial cells, we performed direct binding studies to determine whether or not *alpha*$_2$-adrenergic receptors are present in these cells, and, if so, whether or not clonidine and epinephrine inhibit cyclic AMP accumulation in these isolated cells.

The present report demonstrates that *alpha*$_2$-adrener-

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[2] The abbreviations used are: PGE_1, prostaglandin E_1; VIP, vasoactive intestinal peptide; WB-4101, 2-(2′,6′-dimethoxyphenoxyethylamino)methylbenodioxan; E 643, 2-[4-(*n*-butyryl)-homopiperazine-1-yl]-4-amino-6,7-dimethoxyquinazoline hydrochloride; IBMX, 3-isobutyl-1-methylxanthine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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gic receptors are present in the intestinal epithelial cells, and that stimulation of *alpha*$_2$-adrenergic receptors fails to reduce the cyclic AMP levels in these isolated cells. These results are consistent with our previous suggestion (7) that the antisecretory effect of the *alpha*$_2$-receptor system may be exerted at a step distal to cyclic AMP generation.

EXPERIMENTAL PROCEDURES

Materials. Drugs were donated by the following sources: phentolamine and naphazoline, Ciba-Geigy Company (Takarazuka, Japan); pindolol and dihydroergocryptine, Sandoz Ltd. (Basel, Switzerland); WB-4101, Ward Blenkinsop Company, Ltd. (London, England); prazosin, Taito-Pfizer Company (Tokyo, Japan); azapetine, Hoffmann-La Roche (Basel, Switzerland); tolazoline, Yamanouchi Pharmaceutical Company, Ltd. (Tokyo, Japan); E 643, Eisai Company, Ltd. (Tokyo, Japan); oxymetazoline, Chugai Pharmaceutical Company, Ltd. (Tokyo, Japan); azepexol, Nippon C. H. Sohn Company, Ltd. (Osaka, Japan); (±)-nordefrin, (+)-epinephrine, and (+)-norepinephrine, Sterling-Winthrop (New York, N. Y.); methoxamine, Nippon Shinyaku Company, Ltd. (Kyoto, Japan); (−)-phenylephrine, Kowa Company, Ltd. (Nagoya, Japan); naloxone, Endo Laboratories Inc. (New York, N. Y.). Drugs were purchased from the following sources: yohimbine and phenoxybenzamine, Nakarai Chemical Company (Kyoto, Japan); clonidine, Tokyo Kasei Kogyo Company, Ltd. (Tokyo, Japan); (−)-epinephrine, (−)-norepinephrine, (±)-propranolol, (−)-isoprotelenol, dopamine, PGE_1, and IBMX, Sigma Chemical Company (St. Louis, Mo.); serotonin, Wako pure chemical industries, Ltd. (Osaka, Japan); VIP, Peptide Institute, Inc. (Minoh, Japan); morphine, Sankyo Company, Ltd. (Tokyo, Japan). The [*methyl*-^{3}H]yohimbine (82.6 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.) and was stored at −20°. The [^{3}H]yohimbine solution was diluted with glass-distilled water when added to the binding assay. Its purity was checked routinely by thin-layer chromatography on silica gel plates with two solvent systems: chloroform/diethylamine (9:1, v/v) and methanol/acetic acid (19:1, v/v). The purity of the stock solution was greater than 95%.

Membrane preparation. The intestinal epithelial cells were isolated from rat jejunum and ileum by the method of Stern (12), which was modified by Murer *et al.* (13). In brief, the small intestine was washed with ice-cold physiological saline and then filled with a citrate solution containing 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, and 5.6 mM Na_2HPO_4 (pH 7.3), and incubated for 15 min at 37°. The citrate solution was discarded and the gut was filled with ice-cold buffer containing 250 mM sucrose, 10 mM triethanolamine HCl, and 0.5 mM tetrasodium EDTA (pH 7.6). The filled intestine was gently pressed to release cells into the medium. The cells were collected by centrifugation at 50 × *g* for 5 min. Preliminary experiments showed that both jejunum and ileum possessed similar number of [^{3}H] yohimbine binding sites. In this study, both portions of the small intestine were used. The isolated cells were homogenized with a glass-Teflon homogenizer in the ice-cold triethanolamine buffer (5 ml of buffer per milliliter of packed cells) with 20 strokes at 1500 rpm. The homogenate was added with the equal volume of the buffer and centrifuged at 2,600 × *g* for 15 min at 4°. The resulting supernatant was centrifuged at 100,000 × *g* for 60 min at 4°. The final pellet was resuspended in 83 mM ice-cold Tris-HCl buffer (pH 8.0 at 4°) for use in the binding study. Protein concentrations were determined by the method of Lowry *et al.* (14), using bovine serum albumin as a standard with the appropriate buffer blank.

Standard binding assay. The above freshly prepared tissue extracts (0.9 mg of protein per assay) were incubated with 6 nM [^{3}H]yohimbine, 0.5 mM disodium EDTA, various concentrations of drugs, and 50 mM Tris-HCl buffer (pH 7.5 at 25°) in a total volume of 0.5 ml at 25° for 20 min. When the incubation mixture contained catecholamines, 0.57 mM ascorbate was added in order to prevent the oxidation of the catecholamines. The above concentration of ascorbate had no effect on the [^{3}H] yohimbine binding in the presence of 0.5 mM disodium EDTA. Incubations were terminated by adding 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 8.0 at 4°) followed by rapid filtration through Whatman GF/B glass-fiber filters. The filters were rapidly washed twice with 5 ml of the ice-cold buffer. The total filtration time was less than 5 sec. The filters were placed in scintillation vials with the addition of 7.5 ml of ACS aqueous scintillation mixture (Amersham) and were counted 24 hr later with a liquid scintillation spectrometer at an efficiency of 35%.

Nonspecific binding was defined as those counts observed in the presence of 10 μM phentolamine, a potent *alpha*-adrenergic antagonist that occupies both *alpha*$_1$- and *alpha*$_2$-adrenergic receptor binding sites. Specific binding was defined as total binding minus nonspecific binding and was generally 55% of the total binding.

We ensured that: (a) dilution in 5 ml of ice-cold buffer did not modify the specific binding if followed immediately by filtration; (b) the most reproducible data were obtained with Whatman GF/B glass-fiber filters among the various filters tested; (c) in the absence of tissue, there was no phentolamine-displaceable binding to Whatman GF/B glass-fiber filters.

Measurement of cyclic AMP levels. The collected cells were resuspended in Hanks'-Hepes buffer specified below and centrifuged at 50 × *g* for 5 min. The cells were again resuspended in the same buffer and adjusted to the appropriate concentration. The composition of the Hanks'-Hepes (pH 7.4) buffer was as follows (millimolar): 137 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 0.8 $MgSO_4$, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 5.6 glucose, 4.2 $NaHCO_3$, and 20 Hepes. The cells (0.3 mg of protein per assay) were preincubated at 37° for 5 min in 0.5 ml of the Hanks'-Hepes buffer. After the preincubation, various agents dissolved in a small volume were added to the incubation mixture. The test tube was further incubated at 37° for the time indicated in the figure legends. The viability of cells was checked by the trypan blue exclusion test and was over 90%. The reaction was stopped by immersing the test tube in water at 90° for 5 min followed by the addition of 0.5 ml of glass-distilled water. The contents in the test tube were mixed well and were centrifuged at 900 × *g* for 30 min. An aliquot (100 μl) of the resulting supernatant was taken

for the measurement of cyclic AMP by radioimmunoassay as described elsewhere (6, 7, 11). The remaining pellet was dissolved in 1 N NaOH for the protein assay by the method of Lowry *et al* (14).

Microscopic analyses. For light microscopy, isolated epithelial cells were immersed in 10% formaldehyde and were embedded in paraffin and cut into 10-μm thick sections. Hematoxylin-eosin double-staining procedures were applied to these sections. For electron microscopy, the whole intestinal layer or the isolated cells were fixed for 3 hr in 2.5% glutaralaldehyde in phosphate buffer (pH 7.3, 4°), followed by fixation in 2% OsO_4 in the same buffer at 4° for 2 hr and dehydration in ethanol at room temperature. The specimens were passed through *n*-butyl glycidyl ether and embedded in epoxy resin. They were sectioned with an LKB ultratome, mounted on uncoated 200-mesh grids, stained with 3% uranyl acetate and lead citrate, and examined with a JEM-200CX electron microscope.

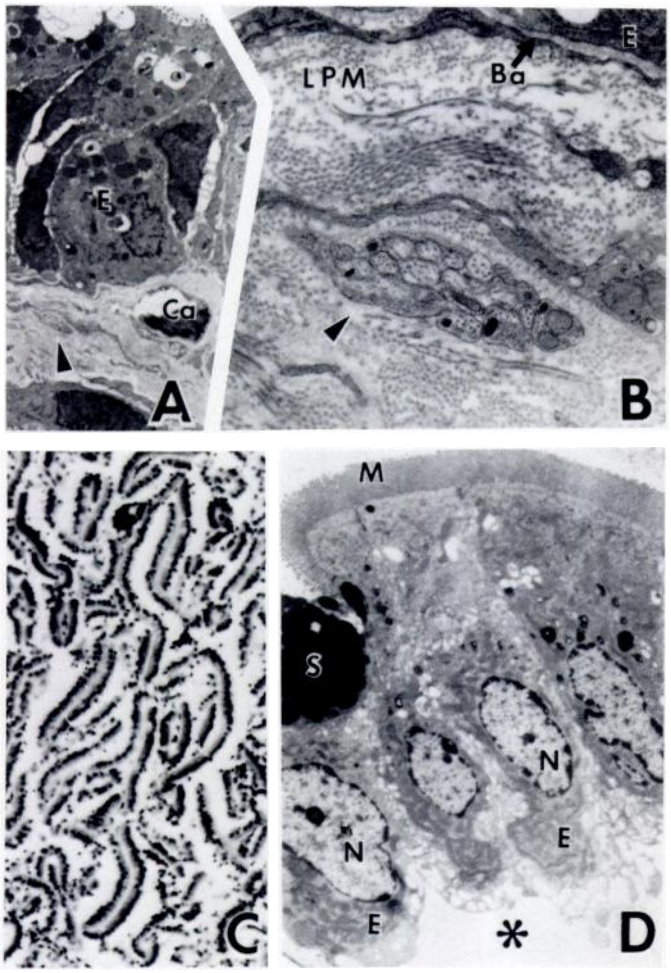

FIG. 1. *Microscopic observations showing the relationship between intestinal epithelial cells and nerve endings*

Observations before (A and B) and after (C and D) the isolation of epithelial cells are shown. A, Below the epithelial cell (*E*), a capillary (*Ca*) and a cluster of nerve endings (*arrowhead*) are noted (×1,600). B, A higher magnification (×13,000) of the *arrowhead* portion of A. The nerve endings (*arrowhead*) are located in the lamina propria mucosa (*LPM*). The basal portion (*Ba*) of the epithelial cell (*E*) are observed above the *LPM*. C, A low-power field (×50) of the preparation of isolated epithelial cells. D, A higher magnification (×2,800) of the cells. Microvilli (*M*), secretory granules (*S*), and nuclei (*N*) of the epithelial cells (*E*) are seen. The *asterisk* shows that no tissues are contaminated with the epithelial cell preparation.

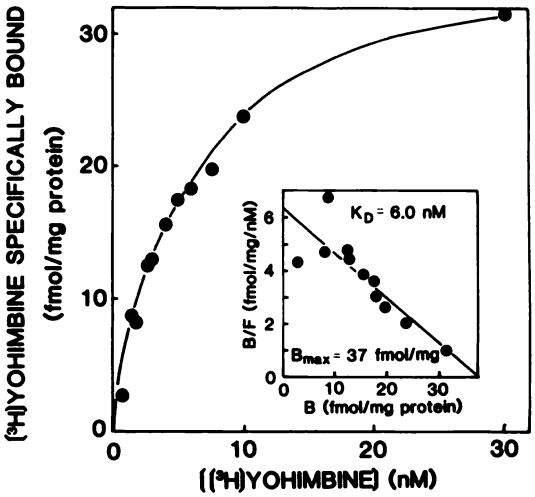

FIG. 2. *[³H]Yohimbine binding as a function of increasing concentrations of [³H]yohimbine*

Indicated concentrations of [³H]yohimbine were incubated with the 100,000 × *g* pellet for 20 min at 25°, as described under Experimental Procedures. Each value is the mean of triplicate determinations, which were replicated four times. [³H]Yohimbine specifically bound was defined as the difference between total and nonspecific binding. *Inset*, Scatchard plot. The ratio (*B/F*) of bound [³H]yohimbine to free [³H]yohimbine is plotted as a function of the concentration of bound [³H]yohimbine (*B*).

RESULTS

Purity of the isolated cells. Figure 1 demonstrates the relationship between the epithelial cells and nerve endings before and after isolation. Figure 1A demonstrates the intestinal epithelial cells and some tissues in the lamina propria before the isolation procedure. Thus, a capillary and a cluster of nerve endings were seen in the vicinity of the basal portion of the epithelial cells. A higher magnification (Fig. 1B) revealed the nerve endings containing secretory granules. The isolated tissues showed the homogeneous morphology in the preparation (Fig. 1C). Electron microscopy of the isolated cells revealed the intestinal epithelial cells and no contamination of the tissues in the lamina propria, such as capillary and nerve endings (Fig. 1D).

Tissue linearity and pH dependency of [³H]yohimbine binding. Specific binding of 6 nM [³H]yohimbine was linear with the concentration of tissue protein in the range of 0.3–2 mg/ml. All assays were conducted within this linear range.

Specific binding at pH greater than 7.5 decreased gradually; the specific binding at pH 9.0 was 30% of the binding at pH 7.5. More acidic condition (<pH 7.5) increased nonspecific binding, and the ratio of specific to nonspecific markedly decreased (data not shown). Therefore, we performed all binding assays at pH 7.5.

Number and affinity of binding sites. Nonspecific binding of [³H]yohimbine increased linearly to 30 nM [³H]yohimbine, the highest concentration examined. The

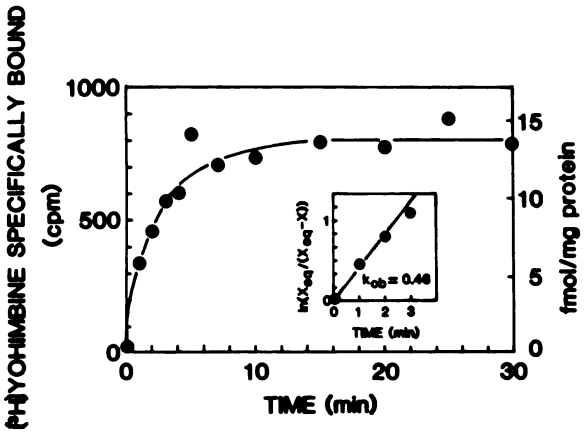

FIG. 3. *Time course of [^{3}H]yohimbine binding*

[^{3}H]Yohimbine (5.3 nM) was incubated with the 100,000 × *g* pellet (1.8 mg of protein per milliliter) at 25° as described under Experimental Procedures and the specific binding was determined at the indicated time. The zero-time value was determined by the addition of [^{3}H]yohimbine immediately (within 3 sec) followed by the filtration procedure. Each value is the mean of triplicate determinations, which were replicated twice. *Inset*, pseudo-first order kinetic plot of [^{3}H]yohimbine binding (r = 0.99). X_{eq} represents the amount of [^{3}H]yohimbine bound at equilibrium, and X the amount of [^{3}H]yohimbine bound at the indicated time. The slope (K_{ob}) is the pseudo-first order rate constant.

specific binding was apparently saturable (Fig. 2). Scatchard analysis (15) indicated a dissociation constant (K_D) of 6.0 nM (correlation coefficient r = 0.85), if one assume a single component of binding sites. The maximal number of binding sites (B_{max}) was about 37 fmoles/mg of protein. A Hill plot (16) revealed a straight line with a Hill coefficient of 0.99 (correlation coefficient r = 0.99), indicating noncooperative interactions.

Kinetics of binding. The rate of binding of [^{3}H]yohimbine was rapid and reached apparent equilibrium within 10 min at 25° (Fig. 3). Because the concentration of radioligand (5.3 nM) was much greater than the binding site concentration in the assay, the forward reaction could be considered a pseudo-first order reaction that depends on the binding site concentration. The slope k_{ob} (Fig. 3), corresponding to the pseudo-first order rate constant, was 0.46 min^{-1} (correlation coefficient r = 0.99).

The rate of dissociation of [^{3}H]yohimbine was examined by incubating the tissue extract to equilibrium and then adding an excess amount of phentolamine in order to prevent rebinding of the dissociated [^{3}H]yohimbine (Fig. 4). At 25°, dissociation was rapid, with a $t_{1/2}$ of about 3 min. If one assumes one component of dissociation, the rate constant for dissociation (k_{-1}) was 0.26 min^{-1}, and the second-order rate constant (association rate constant) k_1 was $k_1 = (k_{ob} - k_{-1})/[YOH] = 3.8 \times 10^7$ M^{-1} min^{-1}. The ratio k_{-1}/k_1 = 6.8 nM is the kinetically derived estimate of the K_D for [^{3}H]yohimbine binding. This value was consistent with the K_D (6.0 nM) obtained from the saturation study (Fig. 2).

Specificity of binding. A number of compounds were

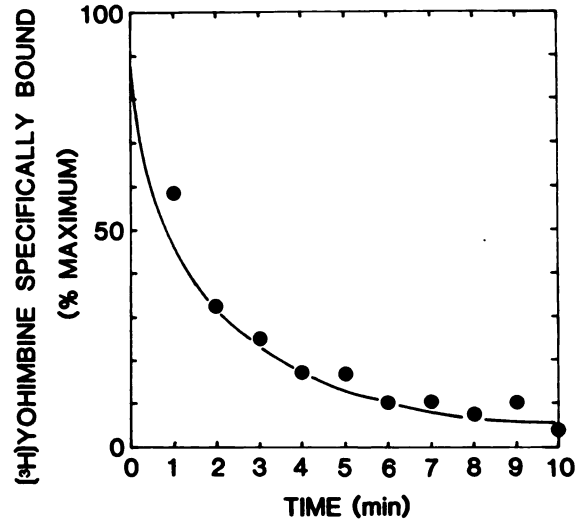

FIG. 4. *Reversibility of [^{3}H]yohimbine binding*

After the 100,000 × *g* pellet was incubated with 5.3 nM [^{3}H]yohimbine at 25° for 20 min, a large excess of phentolamine (10 μM) was added. Phentolamine was added at time zero. Specific binding was determined at the indicated time. Maximal (100%) binding is defined as the amount of binding just prior to the addition of phentolamine. Each value is the mean of triplicate determinations, which were replicated twice.

TABLE 1

IC_{50} of various compounds for inhibiting [^{3}H]yohimbine binding in the 100,000 × g pellet from intestinal epithelial cells

Incubations were performed as described under Experimental Procedures in the absence and presence of four to seven concentrations of the indicated drugs. IC_{50} is the concentration of each agent which caused 50% inhibition of [^{3}H]yohimbine binding. The value is the mean of triplicate determinations, which were replicated twice. The concentration of [^{3}H]yohimbine in the reaction mixture was 6 nM.

Compound	IC_{50}
	μM
Alpha-agonists	
Oxymetazoline	2.0
Clonidine	2.4
Naphazoline	2.8
(±)-Nordefrin	3.9
(−)-Norepinephrine	6.6
Azepexol	7.7
(−)-Epinephrine	8.1
Methoxamine	33
(−)-Phenylephrine	52
(+)-Epinephrine	52
(+)-Norepinephrine	>100
Alpha-antagonists	
Yohimbine	0.011
Dihydroergocryptine	0.013
Phentolamine	0.057
WB-4101	0.52
Azapetine	0.83
Prazosin	1.8
E 643	2.6
Phenoxybenzamine	3.3
Tolazoline	10
Beta-agonist and antagonist	
(−)-Isoproterenol	>100
(±)-Propranolol	14
Others	
Dopamine	>100
Morphine	>100
Naloxone	>100
Serotonin	>100

tested for their abilities to inhibit [^{3}H]yohimbine binding to its binding sites. The order of potency of the adrenergic agonists to inhibit [^{3}H]yohimbine binding was: (−)-norepinephrine > (−)-epinephrine > (−)-phenylephrine ≫ (−)-isoproterenol. This is consistent with the order of potency for the *alpha*-adrenergic receptor, as defined by

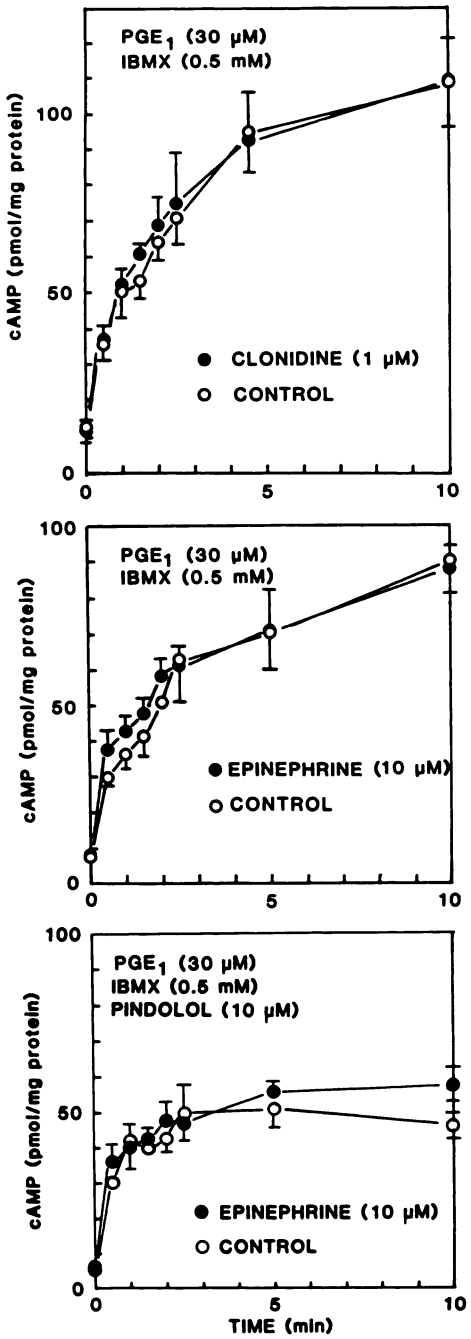

FIG. 5. *Time course of cyclic AMP accumulation induced by PGE$_1$ in isolated intestinal epithelial cells*

The cells were incubated with PGE$_1$ (30 μM) and IBMX (0.5 mM) in Hanks'-Hepes buffer (pH 7.4) at 37° in the presence or absence of 1 μM clonidine (*upper*), 10 μM (−)-epinephrine (*middle*), or 10 μM (−)-epinephrine plus 10 μM pindolol (*lower*). Each value is the mean ± standard error of triplicate determinations. Results qualitatively similar to those shown here were obtained in four other experiments. Where the errors are not indicated, they are within the symbols.

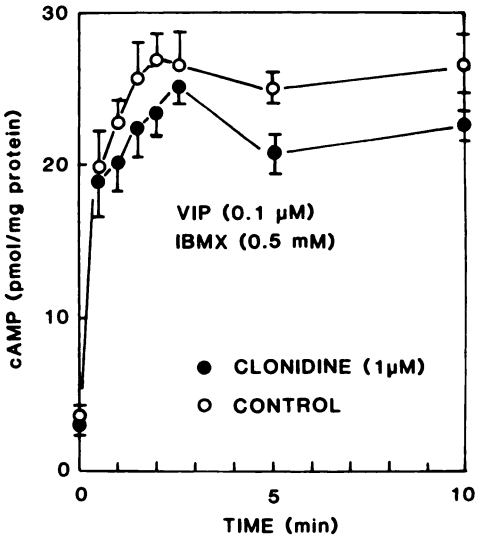

FIG. 6. *Time course of cyclic AMP accumulation induced by VIP in isolated intestinal epithelial cells*

The cells were incubated with VIP (0.1 μM) and IBMX (0.5 mM) in Hanks'-Hepes buffer (pH 7.4) at 37° in the presence or absence of 1 μM clonidine. Each value is the mean ± standard error of triplicate determinations, which were replicated twice. There was no significant difference between two groups.

Ahlquist (17). The binding sites are stereospecific, because the (−)-stereoisomers of epinephrine and norepinephrine were 6-fold and at least 15-fold more potent than the corresponding (+)-stereoisomers, respectively. The specificity of [^{3}H]yohimbine binding site was further confirmed by the binding inhibition studies with adrenergic antagonists. All of the *alpha*-adrenergic antagonists tested showed more potent inhibitory effect than the *beta*-adrenergic antagonist propranolol (Table 1).

The potencies for inhibition of [^{3}H]yohimbine binding shown by the dopaminergic agonist dopamine, the opiate agents morphine and naloxone, and the serotonergic agonist serotonin were all very weak (Table 1).

Table 1 also shows that the *alpha*$_2$-selective agonists oxymetazoline, clonidine, naphazoline, and nordefrin were more potent in inhibiting [^{3}H]yohimbine binding that the *alpha*$_1$-selective agonists phenylephrine and methoxamine. (−)-Norepinephrine, azepexol, and (−)-epinephrine were intermediate between the two extremes. The *alpha*$_2$ specificity was further confirmed by the potency order of *alpha*-antagonists. The *alpha*$_2$-selective antagonist yohimbine was 160-fold more potent in inhibiting [^{3}H]yohimbine binding than *alpha*$_1$-selective antagonists such as prazosin (18) and E 643 (19). The other antagonists indicated have been shown to be nonselective *alpha*-antagonists (18).

Cyclic AMP levels in the isolated intestinal epithelial cells. The basal and stimulated cyclic AMP levels were linearly correlated with cellular protein at least up to 0.3 mg/assay tube. All of the assays were carried out within this linear range. PGE$_1$, VIP, or IBMX alone caused slight but significant stimulatory effects on the cyclic AMP levels (data not shown). PGE$_1$ or VIP simultaneously added with IBMX caused rapid cyclic AMP accumulation (Figs. 5 and 6). The concentration of PGE$_1$ (30

μM) or VIP (0.1 μM) is sufficient to produce maximal cyclic AMP accumulation in the intestinal mucosa (20–22). The concentration (1 μM) of clonidine did not inhibit significantly the cyclic AMP accumulation induced by PGE_1 or VIP. (−)-Epinephrine (10 μM) did not reduce PGE_1-augmented cyclic AMP levels in the absence or presence of a *beta*-adrenergic antagonist, pindolol.

DISCUSSION

The [^{3}H]yohimbine binding satisfied common criteria applied to receptor identification (23, 24): it was saturable, of high affinity, rapid, reversible, stereospecific, and of the pharmacological specificity of *alpha*$_2$-receptors. Because it is known that presynaptic nerve terminals possess *alpha*$_2$-adrenergic receptors (18), the *alpha*$_2$ binding sites are possibly due to the contamination of presynaptic nerve terminals. However, we have excluded this possibility by microscopic observations. Thus, our data provide evidence for the presence of *alpha*$_2$-adrenergic receptors in intestinal epithelial cells. Moreover, the good correlation of the antidiarrheal potency of various agonists with the affinity for *alpha*$_2$-adrenergic receptors (Fig. 7) indicates the involvement of the above receptor mechanism in the inhibition of intestinal secretion.

[^{3}H]Yohimbine has been successfully used for labeling *alpha*$_2$-adrenergic receptors, including human platelets (25–27). We obtained positive and excellent correlation of affinities of various agents for [^{3}H]yohimbine binding sites between human platelets and rat intestinal epithelial cells (results not shown). This may imply that properties of binding sites of the *alpha*$_2$-adrenergic receptors found in the intestinal epithelial cells are close to those in the human platelets. However, the two *alpha*$_2$-receptor systems differ in that, unlike platelet *alpha*$_2$-adrenergic receptors (28), synthetic agents such as clonidine and oxymetazoline produce agonistic effects similar to those of the natural agonist epinephrine (6, 7).

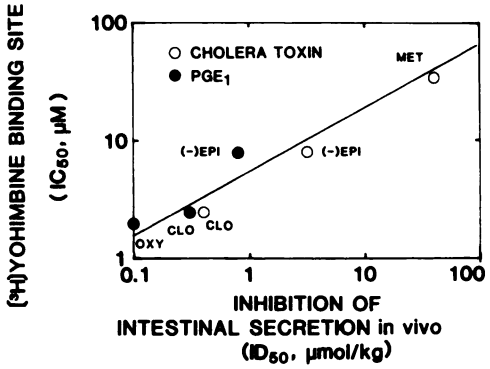

FIG. 7. *Correlation between the IC_{50} values of alpha-adrenergic agonists for [^{3}H]yohimbine binding sites, and the inhibitory effect of the same agents on the cholera toxin- or PGE_1-induced intestinal secretion in vivo*

The IC_{50} values of these agonists were obtained from Table 1. The ID_{50} (micromoles per kilogram) for inhibition of intestinal secretion was obtained from Nakaki *et al.* (6, 7). The intestinal secretion induced by cholera toxin and PGE_1 is shown by ○ and ●, respectively. The correlation coefficient was 0.96 ($p < 0.01$). *OXY*, oxymetazoline; *CLO*, clonidine; *(−)EPI*, (−)-epinephrine; *MET*, methoxamine.

Intestinal secretagogues such as cholera toxin, PGE_1, and VIP cause intestinal secretion with concomitant increases in cyclic AMP levels in the intestinal epithelial cells (4). It has been reported that stimulation of *alpha*$_2$-adrenergic receptors inhibits cyclic AMP accumulation in various tissues (8–11). Our previous studies, however, show that *alpha*$_2$-adrenergic agonists, including clonidine and epinephrine, inhibit secretagogue-induced intestinal secretion without reducing the whole mucosal cyclic AMP levels (6, 7). Consistent with those results, our present data show that clonidine and epinephrine in the absence or presence of the *beta*-antagonist pindolol failed to reduce PGE_1-augmented cyclic AMP levels in isolated epithelial cells. Since these epithelial cells contain *alpha*$_2$-adrenergic receptors (as shown in this communication), the failure of clonidine or epinephrine in the presence of pindolol to reduce PGE_1-augmented cyclic AMP levels is not due to the absence of receptor for the drug. Possible explanations for the discrepancy in the presence of an *alpha*$_2$-receptor and the lack of inhibition of adenylate cyclase by *alpha*$_2$-adrenergic agonists are (a) that the *alpha*$_2$-receptors which we identified are nonfunctioning and are unrelated to the antidiarrheal action of *alpha*$_2$-adrenergic agonists, (b) that the isolated cells are not viable with regard to *alpha*$_2$-receptor systems, and (c) that the *alpha*$_2$-receptors identified in rat intestinal epithelial cells are not negatively coupled with the adenylate cyclase system. The first possibility implies that nonfunctioning receptors were unmasked by homogenization. Since intestinal epithelial cells are densely innervated by noradrenergic neurons (5) and, furthermore, endogenous norepinephrine released from the noradrenergic nerve endings regulates intestinal ion transport (29), this explanation is unlikely. The second possibility arises from the notion that coupling factor(s) between the *alpha*$_2$-receptors and adenylate cyclase were lost during cell preparation. Such an example was recently shown by Stiles and Lefkowitz (30), who demonstrated that trypsin destroys the interface of an "inhibition-specific" guanine nucleotide regulatory unit and the catalytic moiety of adenylate cyclase. However, since no proteolytic enzymes were used in our study, we cannot attribute the observed uncoupling to any recognized factors at present. The third possibility remains; there is no tight interaction between the *alpha*$_2$-adrenergic receptors and the adenylate cyclase in this system.

In contrast to the rat (6, 7), Field *et al.* (20) reported that in the rabbit ileum the *alpha*-adrenergic agonist epinephrine inhibits partially but significantly the increase in total mucosal cyclic AMP levels induced by PGE_1 and cholera toxin. However, since they assayed the cyclic AMP levels of whole mucosal layers, we believe that they were unable to determine whether the reduced mucosal cyclic AMP levels are due to the reduced cyclic AMP levels in the epithelial cells.

According to our previous study (7), clonidine inhibits dibutyryl cyclic AMP-induced intestinal secretion in the rat. This finding is consistent with our present results in that one of the sites of action of *alpha*$_2$-adrenergic agonists on intestinal secretion is probably distal to cyclic AMP generation.

Although *alpha*$_2$-adrenergic receptors are associated

negatively with cyclic AMP accumulation in various systems (8–11), the *alpha*$_2$-adrenergic receptors in the rat intestinal epithelial cells have possibly some biochemical actions other than inhibition of adenylate cyclase.

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