0026-895X/93/010023-07$03.00/0

MOLECULAR PHARMACOLOGY, 43:23-29

Molecular Characterization of α_2-Adrenergic Receptors Regulating Intestinal Electrolyte Transport

KEITH R. HILDEBRAND, GAOFENG LIN, MICHAEL P. MURTAUGH, and DAVID R. BROWN

Department of Veterinary PathoBiology, Pharmacology and Molecular Biology Sections, University of Minnesota College of Veterinary Medicine, St. Paul, Minnesota 55108

Received June 30, 1992; Accepted October 5, 1992

SUMMARY

Norepinephrine (NE) is an important neuromodulator of active Na^+ and Cl^- transport by the small intestine; however, the cellular targets and the adrenergic receptor (AR) subtype mediating its effects on ion transport have not been clearly defined. NE inhibited short-circuit current in submucosal-mucosal sheets of porcine distal jejunum under basal conditions and after electrical transmural stimulation of intrinsic neurons. A membrane fraction (P2) prepared from the submucosa of porcine jejunum was enriched in specific [^{3}H]saxitoxin binding sites, relative to other submucosal fractions. This fraction contained homogeneous and high affinity sites binding the α_2-AR antagonist [^{3}H]yohimbine (K_d = 0.39 ± 0.03 nM). A prazosin versus oxymetazoline K_i ratio of 218 was obtained for the submucosal AR binding site, suggesting that it represents a neuronal α_{2A}-AR. A cell membrane fraction prepared from the mucosa exhibited specific and saturable high affinity binding of the muscarinic cholinergic antagonist [^{3}H] quinuclidinyl benzilate (K_d = 77 ± 9 pM) but displayed minimal specific binding of [^{3}H]saxitoxin or [^{3}H]yohimbine. A [^{32}P]cDNA probe derived from the human α_2-C10 gene encoding the α_{2A}-AR hybridized to a 3.8-kilobase message that was prevalent in poly(A)$^+$ RNA isolated from the jejuno-ileal submucosa and was also detected in porcine cerebral cortex and kidney; no message was detected in RNA isolated from the jejunal mucosa. These results suggest that NE modulates active ion transport in the small intestine through interactions with a submucosal α_{2A}-AR probably associated with enteric neurons.

NE is an important neuromodulator of intestinal water and electrolyte transport (1). It is present in sympathetic neurons whose cell bodies lie outside the gut wall and project to the enteric ganglionated plexuses and enterocytes. In the porcine small intestine, noradrenergic fibers originate in the prevertebral ganglia and terminate in the myenteric and submucosal plexuses (2). On the other hand, noradrenergic fibers that terminate in close apposition to epithelial crypt cells and within the villous core have been described in guinea pig and rat small intestine (3, 4). These anatomical studies suggest that NE may modulate mucosal function by interacting either indirectly with intrinsic neurons projecting to the epithelium or directly with enterocytes.

Catecholamines have been found to modulate active electrolyte transport in the mammalian small intestine, as demonstrated in a number of investigations. In rabbit ileum, catecholamines increase net Na^+ and Cl^- absorption and inhibit Cl^- and HCO_3^- secretion *in vitro* by interacting with epithelial α_2-ARs (5–8). NE also displays anion proabsorptive or antisecretory activities in guinea pig and rat small intestine, as inferred from measurements of transepithelial I_{sc}, a measure of active, electrogenic, ion transport (9–11). NE-induced decreases in I_{sc} are inhibited by the neuronal conduction blocker tetrodotoxin; this finding constitutes evidence that the neurotransmitter affects ion transport in rat and guinea pig small intestine by inhibiting the activity of intrinsic secretomotor neurons (9, 10). It is substantiated by electrophysiological experiments indicating that NE hyperpolarizes submucosal neurons of the guinea pig ileum by activating a transmembrane K^+ conductance (12, 13).

Through measurements of active ion transport in submucosal-mucosal sheets of porcine distal jejunum mounted in Ussing chambers, we have previously found that NE is a potent inhibitor of neurally mediated anion secretion (14). Moreover, NE decreases spontaneous I_{sc} in this preparation by inhibiting tonic neural activity. The effects of NE on neurally evoked mucosal responses were antagonized by yohimbine and mimicked by *p*-aminoclonidine, suggesting that they are mediated through an α_2-AR. The aims of the present investigation were to examine the hypothesis that NE modulates active ion transport by acting on α_2-ARs located on submucosal neurons and to define further the receptor subtype participating in these actions. These aims were accomplished through analysis of

This study was funded in part by grants from the National Institute on Drug Abuse (ADAMHA T32-DA0723407) and the Minnesota Agricultural Experiment Station (MN-061-63). Preliminary reports of this work have appeared (35).

ABBREVIATIONS: NE, norepinephrine; AR, adrenergic receptor; ETS, electrical transmural stimulation; I_{sc}, short-circuit current; PNS, postnuclear supernatant; QNB, quinuclidinyl benzilate; STX, saxitoxin; kb, kilobase(s).

[^{3}H]yohimbine binding to membrane fractions derived from the submucosa and mucosa of the porcine jejunum. Moreover, the differential expression of mRNAs encoding three subtypes of α_2-ARs was examined in these intestinal subregions.

Materials and Methods

Drugs. [^{3}H]STX (47.6 mCi/mg), [^{3}H]yohimbine (247 mCi/mg), and [^{3}H]QNB (147 mCi/mg) were purchased from Amersham Co. (Arlington Heights, IL). Prazosin was a generous gift from Pfizer Ltd. (Sandwich, Kent, England). RX-821002 [2-[2-(2-methoxy-1,4-benzodioxanyl)]imidazoline hydrochloride] and *p*-aminoclonidine were puchased from Research Biochemicals Inc. (Natick, MA). L-Arterenol (NE) hydrochloride and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and tissue isolation. Intestinal tissues were obtained from weaned, outbred, Yorkshire pigs of each sex that were 6 to 10 weeks of age and weighed between 10 and 18 kg. Pigs were housed for 3–7 days in large holding rooms maintained at 25° with constant humidity control and a 12-hr light/dark cycle with lights on at 7 a.m. Pigs had continuous access to water and standard, nonmedicated, pig feed with 18% protein supplement and were not fasted before sacrifice.

At the time of tissue collection, each pig was pretreated with ketamine (10 mg/kg, intramuscularly) and surgical anesthesia was induced with thiamylal (40 mg/kg, intravenously). A midline laparotomy was performed and a segment of distal jejunum (10–20 cm in length) that terminated 5–10 cm proximal to the ileocecal ligament was removed and incised longitudinally along the antimesenteric border. The tissue was placed in ice-cold, oxygenated Ringer-HCO_3 solution modified to approximate porcine extracellular fluid (composition, in mM: Na^+, 148.5; K^+, 6.3; Cl^-, 139.7; Ca^{2+}, 3.0; Mg^{2+}, 0.7; HCO_3^-, 19.6; HPO_4^{3-}, 1.3; $H_2PO_4^{2-}$, 0.3). Anesthetized pigs were euthanized with additional intravenous thiamylal and thoracotomized.

Measurement of ion transport. A portion of the distal jejunum extending bilaterally from the mesenteric attachment was stripped of its serosa and underlying smooth muscle layers by blunt dissection, and the remaining submucosa-mucosa was mounted between two Lucite Ussing-type half-chambers having a flux area of 2.01 cm^2. Although anatomically defined as jejunum in reference to its proximity to the ileocecal ligament, this segment is functionally and histologically similar to the short porcine ileum. Mucosal sheets were bathed in Ringer-HCO_3 solution, maintained at pH 7.4 at 39° (porcine core temperature), and continuously oxygenated (5% CO_2 in O_2) by gas lift. Bathing medium was contained in 10-ml water-jacketed glass reservoirs. D-Glucose and D-mannitol were added to the media bathing the serosal and luminal sides, respectively, of each sheet, to achieve final concentrations of 10 mM. I_{sc} was measured continuously under voltage-clamped conditions as described previously (15); open-circuit transepithelial potential difference was measured at brief time intervals and tissue conductance was calculated by Ohm's law. Rectangular bipolar pulses of electric current (300 pulses at 10 Hz, 0.5-msec pulse duration, 2.8 mA cm^{-2}) were delivered across submucosa-mucosa sheets by a stimulator (model S-88; Grass Instruments, Quincy, MA), as described previously (15). Peak increments in I_{sc} relative to base-line values were determined in response to ETS of secretomotor neurons or drug administration after stabilization of the spontaneous I_{sc} (60–90 min after mounting in Ussing chambers). After the electrically stimulated I_{sc} returned to basal levels (approximately 5 min after the stimulus was terminated), NE was administered to the serosal side of the tissue at a bath concentration of 1 μM. When I_{sc} stabilized in response to NE treatment (approximately 10 min), a second ETS-evoked mucosal response was produced and compared with the initial (control) response. Comparisons between mean mucosal responses to ETS before and after NE treatment were made with a two-tailed paired *t* test.

Membrane preparations. Segments of excised distal jejunum were stripped of their serosa and underlying smooth muscle by blunt dissection. Antimesenteric lymphatic tissue was removed from each segment of distal jejunum. The isolated segment of intestine was then positioned mucosa-side up on the dissecting plate and the mucosa was separated from the submucosa using a razor blade. Submucosal and mucosal tissues were isolated and finely minced, and aliquots of intestinal tissues were stored at −80°.

A submucosal fraction enriched in neuronal cell membranes was prepared after the method of Ahmad *et al.* (16). Briefly, submucosal tissues were homogenized at 25,000 rpm for 30 sec in 10 volumes of ice-cold 50 mM Tris·HCl, pH 7.4, with a Brinkmann Polytron, and the homogenate was centrifuged at 750 × *g* for 10 min. The supernatant was designated the PNS and the pellet was discarded. The PNS fraction was centrifuged at 4000 × *g* for 10 min to yield a pellet (P1) and supernatant (S1). S1 was centrifuged at 48,000 × *g* for 10 min to yield a pellet (P2) and supernatant (S2). The S2 fraction was centrifuged at 120,000 × *g* for 60 min to yield a pellet (Mic 1) and supernatant (S3). The Mic 1 fraction was resuspended in ice-cold Tris·HCl using a hand-held, glass-glass homogenizer and was recentrifuged at 17,000 × *g* for 10 min to yield a pellet (P3) and supernatant (Mic 2). The resulting pellets described above were resuspended in Tris·HCl and stored at −80°. [^{3}H]STX, a ligand that selectively binds to voltage-gated neuronal Na^+ channels, was used to identify membrane fractions isolated from the jejunal submucosa that were enriched in neuronal cell membranes (17).

A cell membrane preparation was made from mucosal scrapings after the method of Laburthe *et al.* (18). Briefly, scrapings were homogenized in ice-cold 50 mM Tris·HCl (pH 7.4) for 1 min at high speed using a Waring blender. The homogenate was centrifuged at 2600 × *g* for 15 min. The pellet was discarded and the supernatant was rehomogenized using a hand-held, glass-glass homogenizer. The supernatant was then centrifuged at 10,000 × *g* for 15 min. The white upper layer of the pellet was resuspended in supernatant using a hand-held homogenizer and was centrifuged at 20,000 × *g* for 20 min. The final pellet containing the plasma membrane-enriched fraction was resuspended in Tris·HCl and stored at −80°. To ensure that the plasmalemma-enriched fraction of the mucosa was isolated and intact, we examined its ability to bind the muscarinic cholinergic antagonist [^{3}H]QNB. Previous studies performed in our laboratory indicated that muscarinic cholinergic ligands affect ion transport processes by interacting with epithelial cell receptors in the porcine distal jejunum (19). Protein determinations were made by the bicinchoninic acid method (20).

Radioligand binding experiments. The binding of [^{3}H]STX and [^{3}H]yohimbine to jejunal membranes was measured in membrane preparations incubated in 50 mM Tris·HCl, pH 7.4, in a total volume of 1.0

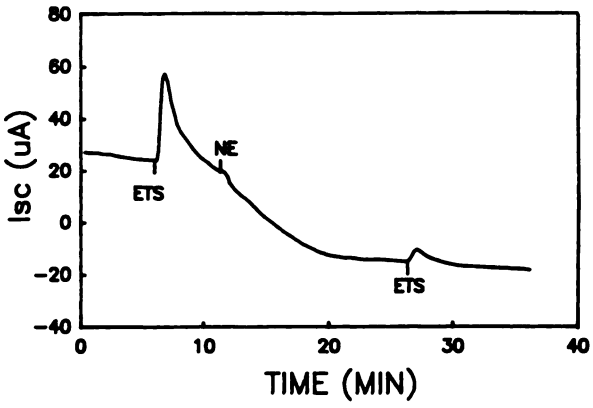

Fig. 1. Effects of NE on basal and electrically evoked I_{sc}. This representative chart record depicts the effects of NE, at a serosal concentration of 1 μM, on basal and ETS-evoked I_{sc} in a sheet of intestinal submucosa-mucosa (surface area, 2.01 cm^2). In this tissue, basal I_{sc} decreased from 11 μA cm^{-2} to −6 μA cm^{-2} in the presence of NE. Moreover, the peak increment in I_{sc} evoked by ETS was 17 and 2 μA cm^{-2} before and after NE addition, respectively. This tracing is representative of responses in 29 tissues obtained from 15 pigs (see Results for mean values).

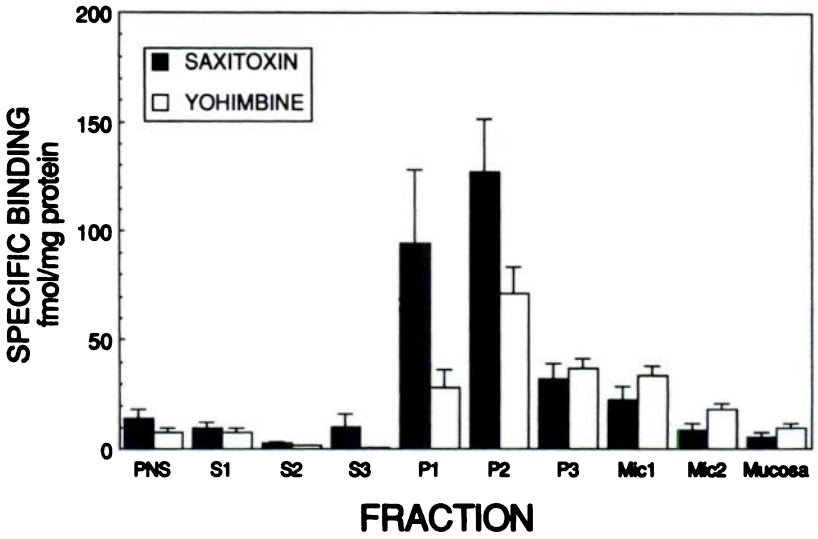

Fig. 2. Specific binding of [^{3}H]STX (1 nM) or [^{3}H]yohimbine (0.2 nM) to membrane fractions isolated from the submucosa and mucosa of the porcine distal jejunum. The P2 fraction of the submucosa exhibited the highest relative specific binding of [^{3}H]STX (127 ± 25 fmol/mg of protein; four experiments peformed in duplicate on four separate batches of homogenate; each batch was prepared from the distal jejunal mucosa or submucosa of three or four pigs). The specific binding of [^{3}H]STX exhibited by the P2 fraction represents a 9-fold enrichment over the specific [^{3}H]STX binding displayed by the PNS. Moreover, the P2 fraction exhibited the highest specific binding of [^{3}H]yohimbine (71 ± 12 fmol/mg of protein), which was 10-fold greater than the specific binding of [^{3}H]yohimbine to the PNS. In contrast to the P2 fraction of the submucosa, the cell membrane-enriched fraction isolated from the mucosa exhibited relatively low specific binding of both [^{3}H]STX and [^{3}H]yohimbine (5 ± 2 and 10 ± 2 fmol/mg of protein, respectively).

ml at 20°. [^{3}H]QNB binding experiments were performed in 50 mM $NaHPO_4$, pH 7.4. Nonspecific binding of [^{3}H]STX, [^{3}H]yohimbine, and [^{3}H]QNB was determined in the presence of the competing unlabeled ligands tetrodotoxin (1 μM), phentolamine (10 μM), and atropine (1 μM), respectively. Radioligands were incubated with membrane suspensions for 45 min ([^{3}H]STX and [^{3}H]yohimbine) or 120 min ([^{3}H]QNB). Reactions were terminated by rapid vacuum filtration on Whatman GF/B filters using a 12-well Brandel cell harvester. Tubes and filters were washed twice with 4 ml of ice-cold Tris·HCl or phosphate buffer and radioactivity was quantified by liquid scintillation counting.

Northern blot analysis. Total RNA was isolated from freshly harvested porcine cerebral cortex, liver, kidney, and distal jejunal mucosa and submucosa by using acid guanidinium thiocyanate extraction (21). Poly(A)$^+$ RNA was enriched from total RNA using one cycle of oligo(dT)-cellulose chromatography (5 Prime-3 Prime, Inc.). Denatured RNA was fractionated by electrophoresis on 2.2 M formaldehyde-1% agarose gels, transferred to Zeta-probe nylon membranes (Bio-Rad) by capillary blotting, and hybridized with random-primed, ^{32}P-labeled, human α_2-C2, α_2-C4, and α_2-C10 cDNA probes, which were generously provided by Dr. Robert Lefkowitz (Department of Biochemistry, Duke University, Durham, NC). The α_2-C2 probe was a 1.35-kb fragment containing the coding sequence from the putative third transmembrane domain through the carboxyl end of the putative third cytoplasmic loop (22). The α_2-C4 probe was a 1.4-kb fragment containing the entire coding block of the human kidney α_2-AR cDNA (23). The α_2-C10 probe was a 5.5-kb fragment containing the coding sequence corresponding to the putative first transmembrane domain through most of the third intracellular loop (24). After hybridization, membranes were washed (for 15 min) successively in 2× standard sodium citrate/0.1% sodium dodecyl sulfate first at room temperature and then at 42°. Membranes were then washed in 0.1× standard sodium citrate/0.1% sodium dodecyl sulfate at 55° for 15–20 min and were exposed to 3M medical X-ray film (type R) at −80° with two intensifying screens. Sizes of RNA bands were estimated by comparison with a 0.24–9.5-kb RNA ladder (BRL Life Technologies, Inc.).

Results

Effects of NE on mucosal ion transport. Under base-line conditions, sheets of submucosa-mucosa isolated from the porcine distal jejunum manifested a serosa-positive I_{sc} of 1 ± 5 μA cm^{-2} and a tissue conductance of 21 ± 1 mS cm^{-2} (29 tissues from 15 pigs). ETS of neurons within mucosal sheets produced rapid increases in I_{sc}, which returned to base-line levels approximately 5 min after the termination of the stimulus (Fig. 1). The peak increment in I_{sc} produced by ETS averaged 53 ± 7 μA cm^{-2}. Mucosal I_{sc} responses to ETS were previously found to be inhibited by tetrodotoxin and the neuronal Ca^{2+} channel blocker ω-conotoxin GVIA (15). Addition of NE to the serosal aspect of mucosal sheets decreased base-line I_{sc} by an average of 18 ± 4 μA cm^{-2} (+1 ± 5 and −18 ± 4 μA cm^{-2} before and after 1 μM NE, respectively; $p < 0.001$, paired t test; 29 tissues from 15 pigs). In addition to its effects on basal I_{sc}, NE decreased the ETS-evoked increment in I_{sc} (53 ± 7 and 4 ± 1 μA cm^{-2} before and after NE, respectively; $p < 0.001$, paired t test; 29 tissues from 15 pigs). In the absence of NE, mucosal responses to the first and second ETS episodes were 48 ± 7 and 45 ± 8 μA cm^{-2}, respectively ($p > 0.05$, paired t test; 16 tissues from eight pigs).

Radioligand binding to membrane fractions from intestinal mucosa and submucosa. Of the nine fractions isolated from the submucosal homogenate, the P2 fraction exhibited the highest relative specific binding of [^{3}H]STX (Fig. 2). This fraction also exhibited the highest relative specific binding of [^{3}H]yohimbine; specific binding represented 80 ± 2% of total binding at the highest concentration (5 nM) of ligand tested (Figs. 2 and 3A). Saturation analysis using [^{3}H]yohimbine was performed to further characterize specific α_2-AR binding sites in neuronal membranes isolated from the submucosa (Fig. 3). The radioligand bound to a single class of binding sites in the P2 fraction (n_H = 1.04 ± 0.02; four saturation experiments, each performed in duplicate, using membranes isolated from the submucosa of six or seven pigs). These sites manifested a high affinity for the radioligand with a K_d of 0.39 ± 0.03 nM and a B_{max} of 358 ± 18 fmol/mg of protein (Fig. 3B).

To test the hypothesis that specific [^{3}H]yohimbine binding sites in the submucosal P2 fraction were α-AR binding sites, experiments were conducted to assess the ability of unlabeled α-AR ligands to compete with [^{3}H]yohimbine for submucosal binding (Table 1). In competition with [^{3}H]yohimbine, AR agonists displayed the relative order of affinity of oxymetazoline > p-aminoclonidine ≫ phenylephrine = NE. Moreover, selective AR antagonists exhibited the relative order of affinity of RX-821002A = yohimbine ≫ prazosin > propranolol (Table 1). Thus, selective α_2-AR ligands exhibited the highest affinities in competing for [^{3}H]yohimbine binding sites. The prazosin

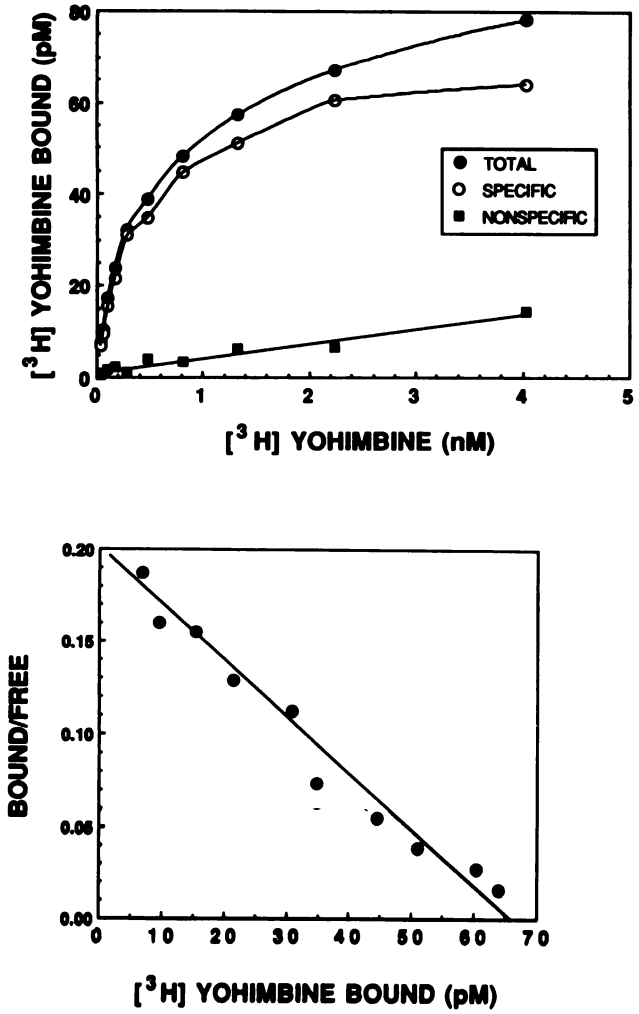

Fig. 3. Saturation analysis of [^{3}H]yohimbine binding to the neuronal membrane-enriched fraction isolated from the submucosa of the porcine distal jejunum. A, Total, nonspecific, and specific binding was determined at each concentration of radioligand. In contrast to the mucosal membranes, the submucosal P2 fraction exhibited relatively high [^{3}H]yohimbine binding, which was predominantly specific in nature (compare Figs. 3A and 4A). B, Representative Scatchard plot determined from the submucosal saturation data shown in A. [^{3}H]Yohimbine bound to the submucosal P2 fraction with high affinity, exhibiting a K_d of 0.34 nM and a B_{max} of 334 fmol/mg of protein in this representative experiment.

versus oxymetazoline and prazosin versus yohimbine K_i ratios were 218 and 980, respectively, which, together with previous pharmacological results, suggests that these sites have characteristics similar to those of α_{2A}-ARs (14).

Cell membranes isolated from jejunal mucosa exhibited relatively low specific binding of both [^{3}H]STX and [^{3}H]yohimbine (Figs. 2 and 4A). In contrast, [^{3}H]QNB bound with high affinity to a single class of sites (K_d = 77 ± 9 pM; B_{max} = 47 ± 5 fmol/mg of protein; n_H = 0.91 ± 0.07) within the mucosal fraction (five saturation experiments, each performed in duplicate, using membranes isolated from six or seven pigs). [^{3}H]QNB binding to these mucosal sites was highly specific, representing 79 ± 2% of total binding observed at a [^{3}H]QNB concentration of 1.6 nM (Fig. 4, B and C).

Identification of RNA species encoding α_2-ARs in porcine intestine and other tissues. In order to examine further the intraintestinal location and characteristics of the α_2-AR mediating the transport-related effects of NE, RNA was isolated from representative porcine tissues including the jejunoileal mucosa and submucosa, analyzed by Northern blotting, and hybridized to cDNA probes specific for each of three identified human α_2-AR subtypes, i.e., α_2-C10, α_2-C2, and α_2-C4 (Fig. 5, A-C). The quality of the poly(A)$^+$ RNA preparation was assessed by hybridization to a porcine β-actin cDNA probe (Fig. 5D). The α_2-C10 probe hybridized to a 3.8-kb mRNA that predominated in the submucosa (Fig. 5A). In contrast, a 3.8-kb hybridization band was not detected in the lane containing poly(A)$^+$ RNA isolated from the jejunal mucosa. Poly(A)$^+$ RNA isolated from cerebral cortex and kidney exhibited low intensity 3.8-kb bands when hybridized to the α_2-C10 probe (Fig. 5A). The α_2-C2 probe hybridized to four different sizes of mRNAs present in the poly(A)$^+$ RNA isolated from porcine tissues. A weak band was detected at 6.4 kb in the kidney and mucosa. Hybridization to a 4.1-kb mRNA predominated in the kidney (Fig. 5B). A weak 4.1-kb band was detected in the jejunal submucosa. This probe also hybridized to a 2.9-kb message that predominated in the kidney but was also present in the cerebral cortex and jejunal mucosa. Hybridization to a 1.2-kb message produced a moderate intensity band in poly(A)$^+$ RNA isolated from kidney and jejunal mucosa (Fig. 5B). The α_2-C4 probe was hybridized to total RNA (50 μg/lane) isolated from porcine tissues (Fig. 5C). This probe hybridized to a 4.1-kb mRNA that was most prevalent in the liver and mucosa; it was also detectable in RNA isolated from the kidney (Fig. 5C). The porcine β-actin probe hybridized to a 2.1-kb mRNA that was present in all four tissue types examined (Fig. 5D). A second 1.5-kb hybridization band was detected in the intestinal mucosa and submucosa.

TABLE 1

Inhibition by selective AR drugs of specific [^{3}H]yohimbine (0.2 nM) binding to neuronal membranes (P2 fraction) isolated from the submucosa of the porcine distal jejunum

Compound	K_i[a]	n_H[b]
	nM	
Agonists		
Oxymetazoline	2.7 ± 0.4	0.801 ± 0.080
p-Aminoclonidine	26 ± 5	0.900 ± 0.028
Phenylephrine	379 ± 76	0.807 ± 0.040
NE	504 ± 161	0.676 ± 0.065
Antagonists		
RX-821002A	0.4 ± 0.1	1.081 ± 0.043
Yohimbine	0.6 ± 0.1	1.040 ± 0.044
Prazosin	588 ± 109	0.811 ± 0.015
Propranolol	2290 ± 188	1.008 ± 0.054

[a] Inhibition constants (K_i) were calculated from the equation $K_i = IC_{50}/(1 + [[^3H]\text{yohimbine}]/K_d)$, where IC_{50} is the concentration of drug displacing 50% of bound radioligand. Each value represents the mean ± standard error of four or five duplicate experiments.

[b] Hill coefficients (n_H) were determined from logit-log plots where the ordinate (logit) = $\ln[P/(100 - P)]$, P = percentage of ligand bound, and the abscissa = logarithm of the molar concentration of the inhibitor.

Discussion

Through the utilization of three distinct experimental approaches (bioelectrical measurements of ion transport, radioligand binding, and tissue-specific detection of RNAs encoding α_2-ARs), we have obtained evidence supporting the hypothesis that an α_{2A}-AR located on submucosal neurons mediates the effects of NE on active electrolyte transport in the small intestine. NE decreased both basal and ETS-evoked I_{sc} in the porcine distal jejunum. A similar inhibitory action of NE has

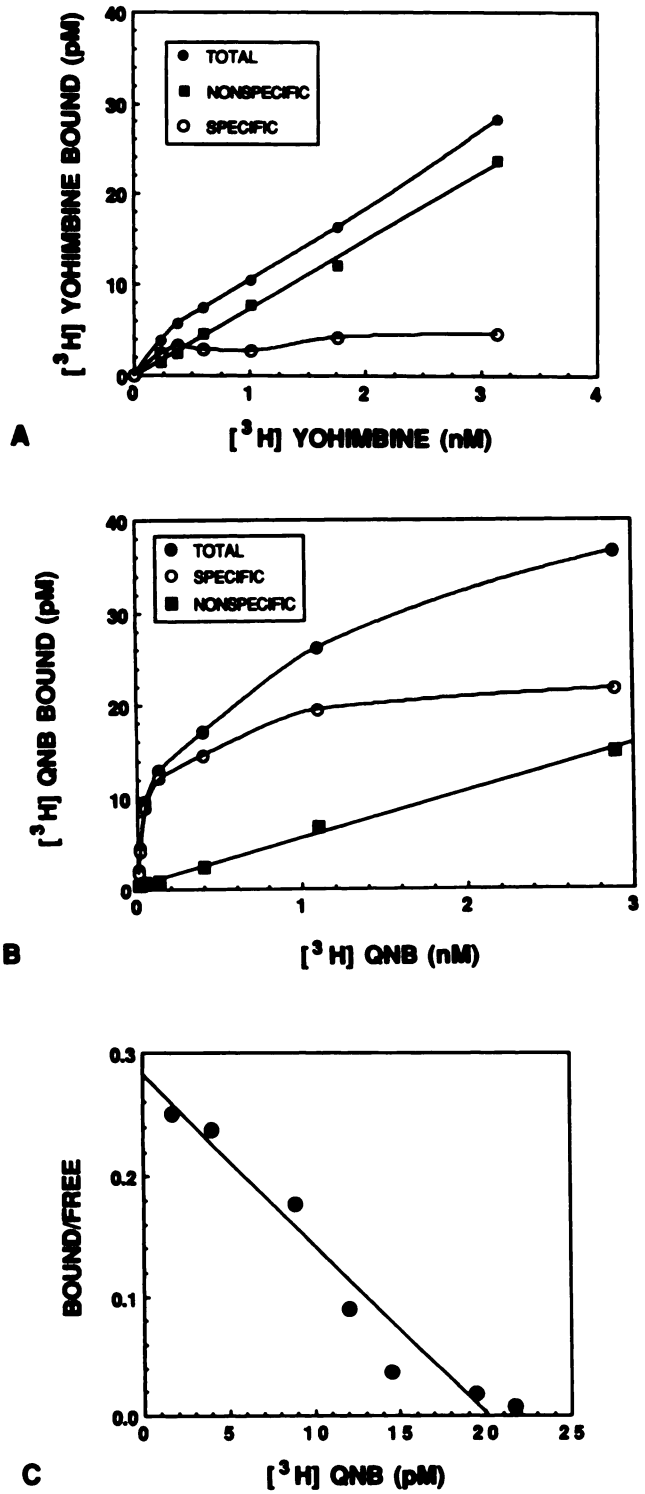

Fig. 4. Saturation analysis of [^{3}H]yohimbine (A) and [^{3}H]QNB (B and C) binding to the plasmalemma-enriched fraction isolated from the mucosa of porcine distal jejunum. Total, nonspecific, and specific binding was determined at each concentration of radioligand. In contrast to the neuronal membranes of the submucosa, the epithelial cell membranes exhibited relatively low [^{3}H]yohimbine binding, which was predominantly nonspecific in nature (compare Figs. 3A and 4A). Despite low specific binding of [^{3}H]yohimbine, the mucosal cell membranes exhibited relatively high specific binding of [^{3}H]QNB (B). C, Scatchard plot determined from the [^{3}H]QNB saturation data. [^{3}H]QNB bound to the mucosal cell membranes with high affinity, exhibiting a K_d of 74 pM and a B_{max} of 50 fmol/mg of protein in this representative experiment.

been documented in rabbit, guinea pig, and rat small intestine as well (5, 9, 10, 25). Depending on the prevailing state of transport processes in this preparation, the inhibition of ongoing neural activity by NE results in net Cl^- absorption or net Na^+ and HCO_3^- secretion; NE-induced inhibition of electrically stimulated neural activity attenuates electrogenic Cl^- and HCO_3^- secretion. These NE actions are mimicked by selective α_2-AR agonists and inhibited by the selective α_2-AR blocker yohimbine (14). In the guinea pig ileum, an α_2-AR linked to hyperpolarization of enteric neurons has been described through single-electrode and whole-cell patch-clamp analyses (9, 12, 13, 26). Furthermore, the rat small intestine appears to possess both neuronal and epithelial α_2-ARs that affect active ion transport, inasmuch as a portion of NE activity is sensitive to neurotoxins (10, 27, 28).

In order to examine in more detail the neuronal α_2-AR mediating transport in porcine small intestine, we prepared a fraction from the porcine jejunal submucosa that was enriched in specific [^{3}H]STX binding sites, indicating the presence of axonal membranes. In canine small intestine, the P2 submucosal fraction likewise exhibits the highest specific [^{3}H]STX binding relative to other submucosal fractions. This latter submucosal fraction also displays high immunoreactivity towards the enteric neuropeptide vasoactive intestinal polypeptide but minimal 5′-nucleotidase activity, suggesting that it consists not only of neuronal membranes but also of intact synaptosomes and that it is relatively free of smooth muscle cell membranes (16). The submucosal P2 fraction of the porcine jejunum possessed the highest relative number of specific [^{3}H] yohimbine binding sites, compared with other membrane fractions. Similarly, the canine submucosal P2 fraction possessed a high density of binding sites specific for [^{3}H]rauwolscine, a diastereoisomer of yohimbine. Ligands selective for α_2-AR competed with [^{3}H]yohimbine for binding to sites within the porcine P2 submucosal fraction with 10–1000-fold greater affinity than the α_1-AR ligands tested. Moreover, the prazosin versus oxymetazoline and the prazosin versus yohimbine K_i ratios that have been used in subtyping of α_2-ARs were 218 and 980, respectively (29, 30). Prazosin versus oxymetazoline and prazosin versus yohimbine K_i ratios that are similar have been obtained in P2 fractions of the canine gut submucosa (16). These results suggest that specific [^{3}H]yohimbine binding sites detected in the porcine submucosa represent an AR of the α_{2A} subtype.

The cell membrane fraction isolated from the porcine jejunoileal mucosa was relatively free of neuronal membrane contamination, as evidenced by minimal specific binding of [^{3}H]STX. Nevertheless, it appeared to be enriched in epithelial cell membranes, inasmuch as the muscarinic cholinergic antagonist [^{3}H]QNB bound saturably and with high affinity to a homogeneous population of specific sites in this preparation. Muscarinic cholinergic agonists stimulate electrogenic anion secretion in the porcine distal jejunum; their effects are resistant to blockade of neural conduction. Moreover, specific [^{3}H]QNB binding sites have been autoradiographically localized within the jejunal wall to the epithelium (19). [^{3}H]Yohimbine displayed relatively low specific binding to this mucosal fraction, in contrast to epithelial membrane fractions from rabbit and rat small intestine, which reportedly exhibit specific binding sites for [^{3}H] yohimbine. The epithelial binding sites characterized in rat and rabbit intestinal mucosa exhibit significantly lower affinity for

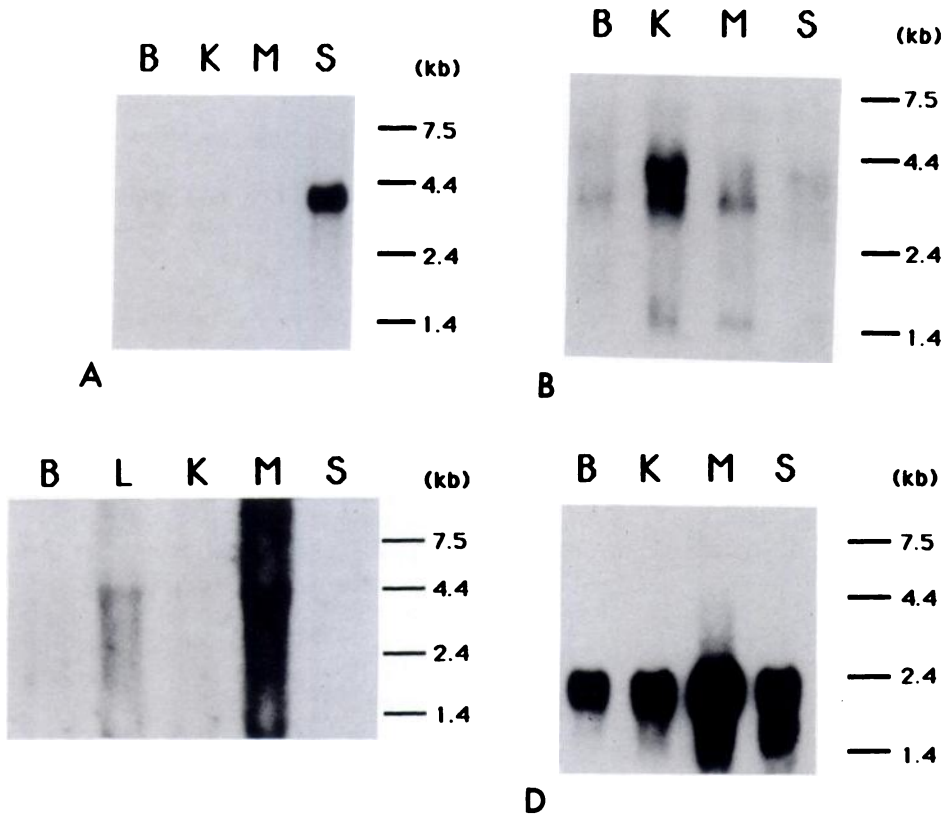

Fig. 5. Northern blot analysis of RNA isolated from porcine brain (cerebral cortex) (*B*), liver (*L*), kidney (*K*), and jejunal mucosa (*M*) and submucosa (*S*). RNA was applied to each lane, electrophoresed, blotted, and hybridized as described in Materials and Methods. The respective tissues are indicated *above each lane*. The sizes of the molecular weight markers are indicated (in kb) to the *right of each blot*. Membranes A, B, and D were prepared by applying 20 μg of poly(A)$^+$ RNA to each lane and hybridizing with human α_2-C10, human α_2-C2, and porcine β-actin DNA probes, respectively. Membrane C was prepared by applying approximately 50 μg of total RNA to each lane and hybridizing with a human α_2-C4 probe. Autoradiograms A, B, C, and D were exposed for 10, 7, 1, and 1 day, respectively.

radioligand (K_d = 6 and 28 nM, respectively) and are at lower densities (B_{max} = 37 and 104 fmol/mg of protein, respectively) than [^{3}H]yohimbine binding sites identified in the porcine submucosa (8, 27).

Northern blot analyses using human cDNA probes for three subtypes of α_2-AR were performed on RNA isolated from the mucosa or submucosa of the porcine distal jejunum, as well as several nonintestinal porcine tissues, to confirm the identity and intramural location of the α_{2A}-AR. Human cDNA probes were utilized in this investigation because of their availability and because of the close amino acid sequence homology (>93%) between the porcine α_{2A}-AR and the human α_2-C10 AR (31). In total, five different species of mRNA were detected in RNA isolated from porcine tissues after hybridization to the three human α_2-AR DNA probes. The α_2-C10 probe is derived from the gene located on chromosome 10 that encodes the α_{2A}-AR (22); it hybridized to a single 3.8-kb species of poly(A)$^+$ RNA that predominated in the jejunal submucosa. This message was also detectable in cerebral cortex and kidney but not the intestinal mucosa. The same α_2-C10 probe has previously been used to identify a 3.8-kb message in rat cerebral cortex and kidney (32). These results, in combination with those from functional and [^{3}H]yohimbine binding experiments, suggest that the gene encoding the α_{2A}-AR is preferentially expressed at the level of mRNA in the submucosa of the intestine and is translated to functional α_{2A}-AR protein.

The α_2-C2 probe, corresponding to the α_{2B}-AR, hybridized to two major species (4.1 and 2.9 kb) of mRNA, both of which were most abundant in the porcine kidney (24, 33). Hybridization of poly(A)$^+$ RNA to the human α_2-C2 probe previously identified a 4.1-kb message in rat kidney and liver (32). Moreover, a different α_{2B}-AR probe derived from rat brain hybridized to both a 4.6-kb message present in rat brain and kidney and a 3.6-kb message present in rat kidney (34). It is likely that the 4.1-kb message identified in porcine kidney represents an α_{2B}-AR. Inasmuch as the porcine submucosa also exhibited a weak 4.1-kb band, it is apparent that at least two subtypes of AR may be present in this tissue. Because functional and binding studies strongly suggest that the AR associated with submucosal neurons is of the α_{2A}-AR subtype, the α_{2B}-AR message may be expressed but not translated or may exist as a receptor protein present on other cell types within this tissue layer that do not mediate the effects of NE on mucosal transport. The α_2-C4 probe, like the α_2-C2 probe, hybridized to a single 4.1-kb message in the total RNA isolated from porcine liver, mucosa, and kidney. This may represent cross-hybridization to the same message identified with the α_2-C2 probe. A 2.9-kb message, representing a third species of α_2-AR mRNA, was identified in the porcine brain, mucosa, and kidney after hybridization to the α_2-C2 probe. Previously, a rat brain α_{2B}-AR probe has been reported to hybridize to a 3.0-kb message also present in rat brain and kidney (34). When rat poly(A)$^+$ RNA was hybridized to the human α_2-C4 probe, a 2.9-kb message was apparent (32). Thus, it is not clear whether this message identified in the pig corresponds to an α_{2B}- or α_{2C}-AR subtype. The human α_2-C4 probe, corresponding to the α_{2C}-AR subtype, did not identify a 2.9-kb message in total RNA isolated from porcine tissues, although this may simply reflect a lower frequency of expression. The α_2-C2 probe also hybridized to 6.4- and 1.2-kb messages present in the porcine kidney and intestinal mucosa. The identity of these messages remains unknown. Thus, unlike the submucosa, the mucosa of the porcine distal jejunum appears to contain four (6.4-, 4.1-, 2.9-, and 1.2-kb) species of mRNA that hybridize to human α_2-C4 and α_2-C2 cDNA probes; a 3.8-

kb α_{2A}-AR mRNA was not detected in this tissue. Because [^{3}H] yohimbine binding sites were not detected on mucosal epithelial cells, these multiple signals may be either expressed as receptors at a low level or expressed in other nonepithelial cell types found in this intestinal subregion.

In summary, our results indicate that an α_{2A}-AR associated with the intestinal submucosa may mediate the actions of NE on active ion transport in the porcine small bowel. This AR may be coupled to a transmembrane K^+ conductance, as described in guinea pig submucosal neurons, or alternatively to a voltage-gated cellular Ca^{2+} conductance (12, 13, 26). The precise mechanisms by which this receptor alters transepithelial ion transport remain to be further defined.

References

1. Brown, D. R., and R. J. Miller. Neurohormonal control of fluid and electrolyte transport in intestinal mucosa. *Handb. Physiol.* **4**:527–589 (1991).
2. Scheuermann, D. W., and W. Stach. Fluorescence microscopic study of the architecture and structure of an adrenergic network in the plexus myentericus (Auerbach), plexus submucous externus (Schabadasch), and plexus submucous internus (Meissner) of the porcine small intestine. *Acta Anat.* **119**:49–59 (1984).
3. Thomas, E. M., and D. Templeton. Noradrenergic innervation of the villi of rat jejunum. *J. Autonom. Nerv. Syst.* **3**:25–29 (1981).
4. Costa, M., and J. B. Furness. Somatostatin is present in a subpopulation of noradrenergic nerve fibres supplying the intestine. *Neuroscience* **13**:911–919 (1984).
5. Field, M., and I. McColl. Ion transport in rabbit ileal mucosa. III. Effects of catecholamines. *Am. J. Physiol.* **225**:852–857 (1973).
6. Field, M., H. E. Sheerin, A. Henderson, and P. L. Smith. Catecholamine effects on cyclic AMP levels and ion secretion in rabbit ileal mucosa. *Am. J. Physiol.* **229**:86–92 (1975).
7. Hubel, K. A. Intestinal ion transport: effect of norepinephrine, pilocarpine, and atropine. *Am. J. Physiol.* **231**:252–257 (1976).
8. Chang, E. B., M. Field, and R.J. Miller. Enterocyte α_2-adrenergic receptors: yohimbine and *p*-aminoclonidine binding relative to ion transport. *Am. J. Physiol.* **244**:G76-G82 (1983).
9. Keast, J. R., J. B. Furness, and M. Costa. Effects of noradrenaline and somatostatin on basal and stimulated mucosal ion transport in the guinea pig small intestine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **333**:393–399 (1986).
10. Butcher, P., M. Hemlin, and H. Sjövall. Neural control of electrogenic transport in the rat jejunum: interaction between intramural and adrenergic mechanisms. *Acta Physiol. Scand.* **131**:235–241 (1987).
11. Cox, H. M., and A. W. Cuthbert. Antisecretory activity of the α_2-adrenoceptor agonist, xylazine in rat jejunal epithelium. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339**:669–674 (1989).
12. North, R. A., and A. Surprenant. Inhibitory synaptic potentials resulting from α_2-adrenoceptor activation in guinea-pig submucous plexus neurones. *J. Physiol. (Lond.)* **358**:17–33 (1985).
13. Tatsumi, H., M. Costa, M. Schimerlik, and R. A. North. Potassium conductance increased by noradrenaline, opioids, somatostatin, and G-proteins: whole-cell recording from guinea pig submucous neurons. *J. Neurosci.* **10**:1675–1682 (1990).
14. Hildebrand, K. R., and D. R. Brown. Norepinephrine and α_2-adrenoceptors modulate active ion transport in porcine small intestine. *J. Pharmacol. Exp. Ther.* **263**:510–519 (1992).
15. Hildebrand, K. R., and D. R. Brown. Intrinsic neuroregulation of ion transport in porcine distal jejunum. *J. Pharmacol. Exp. Ther.* **255**:285–292 (1990).
16. Ahmad, S., H. D. Allescher, H. Manaka, Y. Manaka, and E. E. Daniel. Biochemical studies on opioid and α_2-adrenergic receptors in canine submucosal neurons. *Am. J. Physiol.* **256**:G957-G965 (1989).
17. Ahmad, S., H. D. Allescher, H. Manaka, Y. Manaka, and E.E. Daniel. [^{3}H] Saxitoxin as a marker for canine deep muscular plexus neurons. *Am. J. Physiol.* **255**:G462-G469 (1988).
18. Laburthe, M., B. Chenut, C. Rouyer-Fessard, K. Tatemoto, A. Couvineau, A. Servin, and B. Amiranoff. Interaction of peptide YY with rat intestinal epithelial plasma membranes: binding of radioiodinated peptide. *Endocrinology* **118**:1910–1917 (1986).
19. Chandan, R., K. R. Hildebrand, V. S. Seybold, G. Soldani, and D. R. Brown. Cholinergic neurons and muscarinic receptors regulate anion secretion in pig distal jejunum. *Eur. J. Pharmacol.* **193**:265–273 (1991).
20. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85 (1985).
21. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
22. Kobilka, B. K., H. Matsui, T. S. Kobilka, T. L. Yang-Feng, U. Francke, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Cloning, sequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. *Science (Washington D. C.)* **238**:650–656 (1987).
23. Regan, J. W., T. S. Kobilka, T. L. Yang-Feng, M. G. Caron, R. J. Lefkowitz, and B. K. Kobilka. Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. *Proc. Natl. Acad. Sci. USA* **85**:6301–6305 (1988).
24. Lomasney, J. W., W. Lorenz, L. F. Allen, K. King, J. W. Regan, T. L. Yang-Feng, M. G. Caron, and R. J. Lefkowitz. Expansion of the α_2-adrenergic receptor family: cloning and characterization of a human α_2-adrenergic receptor subtype, the gene for which is located on chromosome 2. *Proc. Natl. Acad. Sci USA* **87**:5094–5098 (1990).
25. Hubel, K. A., K. S. Renquist, and G. Varley. Noradrenergic influence on epithelial responses of rabbit ileum to secretagogues. *Am. J. Physiol.* **256**:G919-G924 (1989).
26. Surprenant, A., K.-Z. Shen, R. A. North, and H. Tatsumi. Inhibition of calcium currents by noradrenaline, somatostatin and opioids in guinea-pig submucous neurones. *J. Physiol. (Lond.)* **431**:585–608 (1990).
27. Nakaki, T., T. Nakadate, S. Yamamoto, and R. Kato. *Alpha*$_2$-adrenergic receptor in intestinal epithelial cells: identification by [^{3}H]yohimbine and failure to inhibit cyclic AMP accumulation. *Mol. Pharmacol.* **23**:228–234 (1983).
28. Cotterell, D. J., K. A. Munday, and J. A. Poat. The binding of [^{3}H]prazosin and [^{3}H]clonidine to rat jejunal epithelial cell membranes. *Biochem. Pharmacol.* **33**:751–756 (1984).
29. Harrison, J. K., W. R. Pearson, and K. R. Lynch. Molecular characterization of α_1- and α_2-adrenoceptors. *Trends Pharmacol. Sci.* **12**:62–67 (1991).
30. Wilson, V. G., C. M. Brown, and J. C. McGrath. Are there more than two types of α-adrenoceptors involved in physiological responses? *Exp. Physiol.* **76**:317–346 (1991).
31. Guyer, C. A., D. A. Horstman, A. L. Wilson, J. D. Clark, E. J. Cragoe, and L. E. Limbird. Cloning, sequencing, and expression of the gene encoding the porcine α_2-adrenergic receptor. *J. Biol. Chem.* **265**:17307–17317 (1990).
32. Lorenz, W., J. W. Lomasney, S. Collins, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Expression of three α_2-adrenergic receptor subtypes in rat tissues: implications for α_2 receptor classification. *Mol. Pharmacol.* **38**:599–603 (1990).
33. Harrison, J. K., D. D. D'Angelo, D. Zeng, and K. R. Lynch. Pharmacological characterization of rat α_2-adrenergic receptors. *Mol. Pharmacol.* **40**:407–412 (1991).
34. Flordellis, C. S., D. E. Handy, M. R. Bresnahan, V. I. Zannis, and H. Gavras. Cloning and expression of a rat brain α_{2B}-adrenergic receptor. *Proc. Natl. Acad. Sci USA* **88**:1019–1023 (1991).
35. Hildebrand, K. R., and D. R. Brown. Characterization of α_2-adrenoceptors modulating active ion transport in porcine distal jejunum. *Pharmacologist* **33**:228 (1991).

Send reprint requests to: Keith R. Hildebrand, D.V.M., Department of Veterinary PathoBiology, University of Minnesota, College of Veterinary Medicine, 1988 Fitch Avenue, St. Paul, MN 55108.