

Pharmacological Evidence for a 7-Benzylidenenaltrexone-Preferring Opioid Receptor Mediating the Inhibitory Actions of Peptidic δ - and μ -Opioid Agonists on Neurogenic Ion Transport in Porcine Ileal Mucosa

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ABSTRACT

The antidiarrheal and constipating effects of opiates are partly attributed to reductions in active anion secretion across the intestinal mucosa that are modulated by submucosal neurons. In this study, the opioid receptor mediating the actions of opioids on ion transport was characterized in mucosa-submucosa sheets from porcine ileum. Electrical transmural stimulation evoked transient increases in short-circuit current, an electrical measure of neurogenic ion transport, in this preparation. After serosal addition, the peptidic δ -opioid agonists [D-Ala²]-deltorphin II (pIC₅₀ = 8.4 ± 0.7), [D-Ala²,D-Leu⁵]-enkephalin (DADLE), [D-Pen²,D-Pen⁵]-enkephalin (DPDPE), and [D-Ser²,Leu⁵,Thr⁶]-enkephalin (DSLET), and the μ -opioid agonists [D-Ala²,N-methyl-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) (pIC₅₀ = 8.0 ± 0.1), endomorphin I, and PL-017 inhibited short-circuit current elevations. Nonpeptidic μ - or δ -opioid agonists (morphine, loperamide, and SNC80) and κ -opioid agonists (U-

50,488H and U-69,593) were <360-fold less potent than deltorphin II. At 100 nM, the δ ₁-opioid antagonist 7-benzylidenenaltrexone reduced the potencies of DPDPE and DAMGO by 13.5- and 15.5-fold, respectively; at an identical concentration naltriben, a δ ₂-opioid antagonist, or the μ -opioid antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) reduced DPDPE potency by 4.1- and 3.4-fold, respectively, but had no significant effect on DAMGO potency. Using primary antisera directed toward cloned opioid receptors, δ -opioid receptor immunoreactivity was immunohistochemically localized in submucosal neurons and nerve fibers, but immunoreactivities to κ - or μ -opioid receptors were not detected in the mucosa-submucosa. These results suggest that a novel 7-benzylidenenaltrexone-sensitive opioid receptor is expressed in submucosal neurons of the porcine ileum, which mediates the inhibitory effects of peptidic μ - and δ -opioid agonists on neurogenic ion transport.

The active secretion of chloride and bicarbonate ions across intestinal epithelial cells is crucial in maintaining an aqueous environment for digestive processes and as a primary defense mechanism against infection. Opiate alkaloids and endogenous opioid peptides reduce intestinal secretion and promote absorption of salt and water, and these actions contribute to the well known antidiarrheal and constipating effects of these substances. The intestinal antisecretory actions of opioids are predominately mediated by δ -opioid receptors (δ -ORs) present on neurons within the central and enteric nervous systems (Brown

and Miller, 1991). In the small intestine, opioids interacting at δ -ORs on submucosal neurons inhibit neurotransmission by increasing K⁺ conductance and decreasing Ca²⁺ conductance (Mihara and North, 1986; Surprenant et al., 1990). Furthermore, the antisecretory actions of opioids are mediated by δ -ORs in isolated mucosa-submucosa sheets from guinea pig, murine, and porcine small intestine (Kachur and Miller, 1982; Sheldon et al., 1990; Quito and Brown, 1991). Intestinal opioid receptors may also be present on enterocytes in some animal species and intestinal segments, although their physiological relevance in the modulation of epithelial transport is controversial (Gaginella et al., 1983; Dashwood et al., 1985; Lang et al., 1996; Nano et al., 2000).

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ABBREVIATIONS: OR, opioid receptor; DADLE, [D-Ala²,D-Leu⁵]-enkephalin; DPDPE, [D-Pen²,D-Pen⁵]-enkephalin; DSLET, [D-Ser²,Leu⁵,Thr⁶]-enkephalin; DAMGO, [D-Ala²,N-methyl-Phe⁴,Gly⁵-ol]-enkephalin; PL-017, [methyl-Phe³,D-Pro⁴]morphiceptin; TIPP, Tyr-1,2,3,4-tetrahydroisoquinoline-Phe-Phe-OH; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂; SNC80, (+)-4-[(α R)- α (2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl-3-methoxybenzyl]-N,N-diethylbenzamide; U-50,488H, *trans*-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl) benzene-acetamide methanesulfonate; U-69,593, (5 α ,7 α ,8 β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzeneacetamide; NTB, naltriben; BNTX, 7-benzylidenenaltrexone; BSINTX, 7-(5',6'-benzo-2'-spiro-indanyl)naltrexone; I_{sc}, short-circuit current; G_t, tissue electrical conductance; ETS, electrical transmural stimulation; PBS, phosphate-buffered saline.

Although pharmacological evidence supporting the existence of at least two putative δ -OR subtypes has accumulated over the past decade, complementary DNA encoding only one δ -OR cDNA has been cloned (Quock et al., 1999). Receptors of the δ_1 subtype have been so classified by their preferential affinity for the δ -OR agonists DADLE and DPDPE, and the competitive and noncompetitive δ -OR antagonists 7-benzylidenenaltrexone and [D-Ala²,Leu⁵,Cys⁶]-enkephalin, respectively. On the other hand, the δ -OR agonists DSLET and deltorphin II have higher potency at the putative δ_2 -OR, as do the respective competitive and nonequilibrium δ -OR antagonists naltriben and naltrindole 5'-isothiocyanate (Zaki et al., 1996). The mouse vas deferens, a common bioassay preparation for the evaluation of drug activity at δ -ORs, expresses a single δ -OR, which does not clearly distinguish ligands with putative selectivity for δ_1 - or δ_2 -OR subtypes (Wild et al., 1993). The cloned δ -OR also does not distinguish between δ -OR subtype-selective ligands (Knapp et al., 1995; Clark et al., 1997). Adding to the complexity of categorizing these receptors, δ -ORs have frequently been found to interact with μ -ORs, and this phenomenon has led to the concept that δ - and μ -ORs may be complexed in some neural pathways (Rothman et al., 1988). Finally, it has been reported that μ - and δ -ORs can form a functional heterodimeric receptor when recombinantly expressed in cultured cells (George et al., 2000). Naturally occurring μ/δ -heterodimeric ORs have not so far been identified.

We have previously reported that DPDPE and DAMGO, highly selective agonists for δ - and μ -ORs, respectively, decrease neurogenic anion secretion evoked by electrical transmural stimulation of mucosal sheets from the porcine distal jejunum. They do so with similar potencies and effectiveness, and the actions of both agonists are inhibited by the selective δ -OR antagonists naltrindole and ICI-174, 864 (Quito and Brown, 1991). The present investigation was conducted to extend these initial observations to the porcine ileum and characterize in more detail the pharmacological characteristics of the opioid receptor(s) mediating the inhibitory actions of μ - and δ -OR agonists on neurogenic ion transport. Furthermore, the presence and distribution of ORs in submucosal neurons and nerve fibers was examined by immunohistochemical techniques using antisera raised against epitopes in cloned δ -, μ -, and κ -ORs.

Materials and Methods

Drugs and Reagents. [D-Ala²,N-methyl-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO), [D-Pen²,D-Pen⁵]-enkephalin (DPDPE), [D-Ala²,D-Leu³]-enkephalin (DADLE), [D-Ser²,Leu⁵,Thr⁶]-enkephalin (DSLET), [D-Ala²]-deltorphin II, [methyl-Phe³,D-Pro⁴]morphiceptin (PL-017), Tyr-(1,2,3,4-tetrahydroisoquinoline-3-carboxylate)-Phe-Phe-OH (TIPP), and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Orn-Thr-Pen-Thr-NH₂ (CTOP) were obtained from Peninsula Laboratories, Inc. (Belmont, CA). (+)-4-[(α R)- α -(2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl-3-methoxybenzyl]-N,N-diethylbenzamide (SNC80) was purchased from Tocris Cookson (Ballwin, MO), and *trans*-(\pm)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide methane-sulfonate (U-50,488H), naloxone, and (5 α ,7 α ,8 β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzeneacetamide (U-69593) were obtained from Research Biochemicals International (Natick, MA). Naltriben (NTB), 7-benzylidenenaltrexone (BNTX), and 7-(5',6'-benzo-2'-spiro-indanyl)naltrexone (BSINTX) were synthesized in the laboratory of P.S.P. as previously reported (Portogh-

ese et al., 1991; Korlipara et al., 1994; Ohkawa et al., 1997). All other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Peptides were solubilized in 0.01 M acetic acid with 0.1% bovine serum albumin, aliquoted at stock concentrations of 1 to 10 mM, and stored until use at -20°C . U-50,488H and U-69,593 were solubilized in 45% (w/v) aqueous 2-hydroxypropyl- β -cyclodextrin before use. Stock solutions of SNC80 and loperamide hydrochloride were made in 100 mM HCl and 50% aqueous methanol, respectively; subsequent serial dilutions were made with distilled water. All other drugs and chemicals were dissolved in distilled water. Mucosal responses were not altered by any of the diluted solvents used in these experiments.

Animals. Intestinal tissues were obtained from Yorkshire pigs (6–10 weeks of age; 10–18 kg of body weight) of each sex, which were not fasted before sacrifice. Animals were sedated with an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol, 8 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA), in combination with xylazine (8 mg/kg). The animals were subsequently euthanized by barbiturate overdose in accordance with approved University of Minnesota Animal Care Committee protocols. A midline laparotomy was performed to expose the intestine and a portion of the ileum, identified by its attachment to the ileo-cecal ligament, was removed and placed in an oxygenated physiological salt solution approximating the composition of porcine extracellular fluid (composition 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 0.5 mM MgCl₂, 25 mM NaHCO₃, 1.0 mM NaH₂PO₄, and 11 mM D-glucose; pH 7.4).

Measurement of Epithelial Ion Transport. Ileal segments were stripped of their smooth muscle coats and sheets of mucosa-submucosa were mounted between two Lucite half-chambers (Jim's Instrument Manufacturing Co., Iowa City, IA) having a flux area of 2 cm². Immunohistochemical examination of these tissues with an antibody to the neuronal marker protein gene product 9.5 (see below) revealed that they contained the inner submucosal ganglia, but outer submucosal ganglia could only be visualized occasionally. Approximately 78% of nerve fibers innervating the porcine small intestinal mucosa originate from neurons in the inner submucosal ganglia (Hens et al., 2000). Mucosal sheets were bathed on both sides with the physiological salt solution described above at pH 7.35 and gassed with 5% CO₂ in O₂ at 39°C (porcine core temperature). D-Glucose and mannitol were added to the serosal and luminal media at 10 mM, respectively. The short-circuit current (I_{sc}) across the tissues, a measure of net ion transport, was monitored continuously by an automatic voltage clamp (Model TR100; JWT Engineering, Overland Park, KS) with the serosal side as reference. After the basal I_{sc} had stabilized, the circuit was frequently opened throughout each experiment to obtain transepithelial potential difference (mV) to calculate tissue conductance (G_t in mmho/cm²) according to Ohm's law. After an equilibration period, electrical transmural stimulation (ETS; 300 bipolar current pulses at 10 Hz., 0.5-ms pulse duration, 2.1 mA/cm²) was delivered by a Model S-88 stimulator and Model SIU-5 stimulus isolation unit (Grass Instruments, Quincy, MA) connected to aluminum foil electrodes placed diagonally on opposite sides of mucosa-submucosa sheets. After three successive rounds of ETS-produced I_{sc} elevations of equal magnitude, drugs were added at increasing concentrations to the serosal aspect of tissues 5 min before delivery of ETS. Ten minutes before agonist addition, some tissues were exposed to antagonists at a serosal concentration of 100 nM. Changes in ETS-evoked peak I_{sc} elevations produced after drug administration were measured and compared with responses to ETS before drug addition.

Immunohistochemistry. Muscle-stripped sheets of ileal mucosa-submucosa from five pigs identical to those used in the pharmacological experiments described above were cut in blocks of 1 to 2 cm² and immersed in ice-cold 2% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 2 h. The tissues were then cryoprotected in graded (10–30%) concentrations of sucrose in PBS, embed-

ded in TissueTek O.C.T. compound (Baxter Healthcare Corp., McGaw Park, IL), and frozen. Longitudinal or transverse cryostat sections (14 μm in thickness) were thaw-mounted onto Superfrost-plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -20°C until use. Tissues were rehydrated in PBS for 15 min and preincubated in PBS containing 0.4% Triton X-100 (Sigma Chemical Co.) and 3% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) in PBS for 30 min at room temperature to block nonspecific binding. Sections were incubated overnight at 4°C with one or more of primary anti-OR antibodies at 1:1000 dilutions referenced in Table 1. An antibody against the neuronal marker protein gene product 9.5 (1:150 dilution; Chemicon International Inc., Temecula, CA) raised in rabbits was used in adjacent sections to confirm neuronal morphology. All antibodies were diluted in 0.4% Triton X-100 and 3% normal donkey serum. Sections were washed in PBS for 15 min, and then incubated with appropriate secondary antibodies [donkey anti-rabbit indocarbocyanine 3-conjugated IgG at 1:400 dilution or donkey anti-goat fluorescein isothiocyanate-conjugated IgG at 1:40 dilution] in PBS for 1 h in the dark. Sections were subsequently washed in PBS for 15 min, coverslipped with Vectashield (Vector Laboratory, Burlingame, CA), and stored at -20°C . Control experiments included the omission of primary antibodies from the staining protocol or the preabsorption of primary antibodies with their relevant blocking peptides in 100-fold molar excess. Although preabsorption of primary antibodies resulted in the complete absence of immunoreactivity in neural elements, some nonspecific staining persisted in mucosal epithelial and lymphoid cells.

Tissue sections were scanned using a Bio-Rad confocal laser scanning microscope (model 1024), which was attached to a Nikon fluorescence microscope. Images were obtained using Comos software (version 6.05.8; Comos Bio-Rad, Hercules, CA) and further processed employing NIH Image (version 1.59) and Adobe Photoshop (version 4.0).

Data Analysis. Opioid-induced changes in ETS-induced peak I_{sc} elevations are expressed as percentage of inhibition of predrug peak I_{sc} amplitude (mean \pm S.E. of concentration-effect determinations in n tissues from N pigs). Determinations of agonist concentration-effect relationships through nonlinear regression methods and statistical analyses of data were performed using the PRISM computer software program (version 2.0; GraphPad Software, Inc., San Diego, CA). Antagonist equilibrium constants (K_e) were calculated according to the method of Kosterlitz and Watt (1968). Agonist potencies are expressed as the negative logarithm of the 50% inhibitory concentration (pIC_{50}) and this parameter was used in all statistical comparisons of agonist potency. Comparisons between a single control and treatment mean were made by paired or unpaired two-tailed Student's t tests when appropriate. Comparisons of a control mean with multiple treatment means were made by analysis of variance followed by Dunnett's test; Tukey's test was used to make comparisons among the mean pIC_{50} values for opioid agonists. In all cases, the limit for statistical significance was set at $P < 0.05$.

TABLE 1
Description of anti-opioid receptor antibodies used in immunohistochemical experiments

Antibody	Host Species	Epitope Sequence	Relevant Sequence in Porcine Opioid Receptor ^a (GenBank accession number)
DOR-461 ^b	Rabbit	Amino acids 103–120 (mouse δ -OR) PFQSAKYLMETWPFGEELL	PFQSAKYLMETWPFGEELL (U71149)
MOR-1 (N–20) ^c	Goat	Amino acids 1–20 (human μ -OR) MDSSAAPTNASNCTDALAYS	MDSSADPRNASNCTDPFSPS (L38645)
KOR-1 (N–19) ^c	Goat	Amino acids 1–19 (human κ -OR) MESPIQIFRGEFGPTCAPSA	Unknown

^a Sequences for porcine δ - and μ -opioid receptors are, respectively, cited in Brown et al. (1998) and Pampusch et al. (1998).

^b Generous gift from Dr. Robert P. Elde, Department of Neuroscience, University of Minnesota-Twin Cities (Arvidsson et al., 1995).

^c Purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

Results

Electrically Evoked Short-Circuit Current Changes in Ileal Mucosa-Submucosa Sheets. ETS produced transient elevations in I_{sc} of $66.0 \pm 2.5 \mu\text{A}/\text{cm}^2$ ($P < 0.05$, paired t test, $n = 152$ tissues from 67 pigs) and increases in G_t averaging $3.2 \pm 1.3 \text{ mmho}/\text{cm}^2$ ($P < 0.05$, paired t test, $n = 122$ tissues from 62 pigs) relative to baseline values. In the absence of drugs, the baseline I_{sc} or ETS-induced I_{sc} did not change significantly [$P > 0.05$ for both parameters, one-way analyses of variance, $F(55,69) = 0.06$ and 0.37 , respectively] over the 90-min experimental period. In addition, G_t did not change significantly over this time period (data not shown).

Mucosal I_{sc} responses to ETS have previously been shown to be abolished by the neuronal Na^+ channel blocker tetrodotoxin (Hildebrand and Brown, 1990). Similarly, a related neuronal conduction inhibitor, saxitoxin, at a serosal concentration of 100 nM, inhibited responses to ETS by $70 \pm 9\%$ ($P < 0.01$ versus predrug condition, paired t test, four tissues from four pigs). Serosal addition of the ganglionic blocker hexamethonium (10 μM) also inhibited ETS-evoked increases in I_{sc} by $59 \pm 6\%$ ($P < 0.01$ versus predrug condition, paired t test, four tissues from four pigs).

Effects of Selective Opioid Receptor Agonists on Electrically Evoked Mucosal Ion Transport. All OR agonists attenuated ETS-evoked peak increases in I_{sc} in a concentration-dependent manner after their addition to the serosal bathing medium (Fig. 1). Deltorphin II and other peptidic δ -OR agonists were among the most potent substances tested, producing half-maximal inhibitory effects at nanomolar concentrations with an order of potency of deltorphin II \geq DADLE \geq DPDPE $>$ DSLET (Fig. 1). They were >89 -fold more potent in decreasing mucosal I_{sc} responses to ETS than the synthetic δ -OR agonist SNC80 (Table 2).

Peptidic μ -OR agonists inhibited neurogenic ion transport with potencies that were not significantly different from that of deltorphin II; they reduced mucosal I_{sc} responses to ETS with an order of potency of DAMGO \geq endomorphin I \geq PL-017 (Fig. 1). They were >38 -fold more potent than the nonpeptidic μ -OR agonists morphine and the meperidine-related antidiarrheal drug loperamide (Table 2).

Of the OR agonists tested, the two κ -OR agonists U-69,593 and U-50,488H had the lowest potencies (Fig. 1). U-69,593 was approximately 30% less effective than U-50,488H in reducing ETS-evoked I_{sc} elevations (Table 2).

To determine whether a functional interaction between δ - and μ -OR agonists exists, the effect of DPDPE on morphine

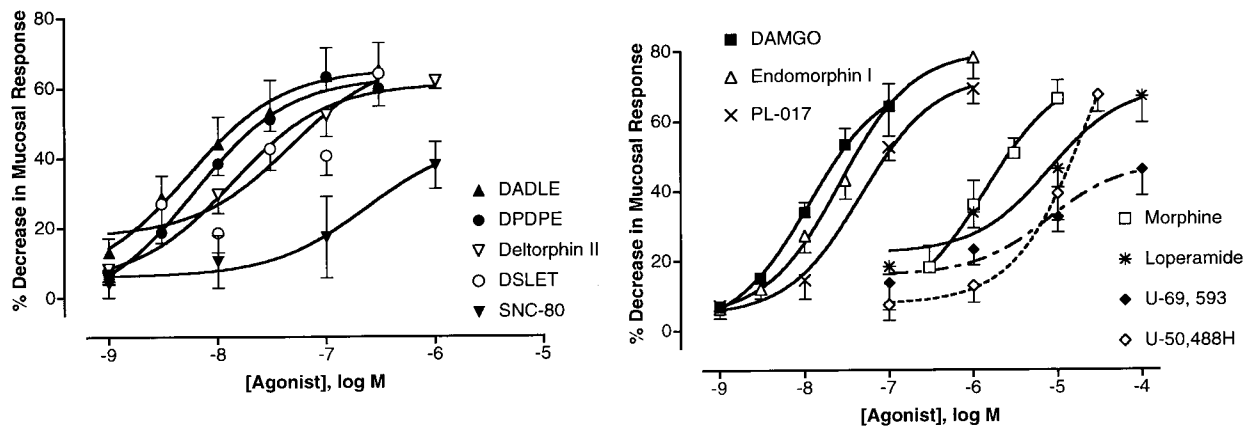


Fig. 1. Concentration-dependent actions of opioid receptor agonists in inhibiting neurogenic ion transport in porcine ileal mucosa-submucosa sheets. Left, concentration-effect relationships for δ -opioid agonists in attenuating ETS-evoked increases in I_{sc} . Each point represents the mean \pm S.E.M. of responses in 3 to 15 mucosal sheets from 3 to 15 pigs. Right, concentration-effect relationships for μ - and κ -opioid agonists in attenuating ETS-evoked increases in I_{sc} . Each point represents the mean \pm S.E.M. of responses in 4 to 11 mucosal sheets from 3 to 11 pigs.

TABLE 2

Relative potencies and maximum inhibitory effects of selective opioid agonists on neurogenic ion transport

Agonist	Receptor Activity	pIC ₅₀	n Tissues (from N Pigs)	E _{max}	Relative Inhibitory Activity ^a
<i>M</i>					
Deltorphan II	δ_2	8.37 \pm 0.72	5 (5)	67.8 \pm 2.2	1.00
DADLE	δ_1	8.15 \pm 0.23	4 (4)	65.2 \pm 7.6	0.96
DPDPE	δ_1	8.11 \pm 0.08	15 (15)	63.1 \pm 3.8	0.93
DAMGO	μ	8.00 \pm 0.07	11 (11)	64.2 \pm 6.5	0.95
DSLET	δ_2	7.60 \pm 0.33	11 (9)	64.1 \pm 5.0	0.95
Endomorphin I	μ	7.54 \pm 0.15	5 (3)	78.1 \pm 6.2	1.15
PL-017	μ	7.39 \pm 0.12	4 (3)	69.2 \pm 4.4	1.02
Morphine	μ	5.81 \pm 0.11**	5 (5)	66.2 \pm 5.4	0.98
SNC80	δ	5.65 \pm 1.11**	3 (3)	37.7 \pm 6.7	0.56
Loperamide	μ, δ	5.51 \pm 0.16**	4 (3)	66.9 \pm 7.6	0.99
U-69,593	κ	5.39 \pm 0.48**	4 (4)	45.9 \pm 7.4	0.68
U-50,488H	κ	4.55 \pm 0.26**	4 (3)	67.3 \pm 4.9	0.99

** $P < 0.01$ versus [D-Ala²]-deltorphan II pIC₅₀ value, Dunnett's test; 12,66 df.

^a Inhibitory effectiveness relative to [D-Ala²]-deltorphan II.

activity was examined. At a submaximal serosal concentration of 3 nM, DPDPE did not significantly change the potency or effectiveness of morphine (morphine pIC₅₀ in the presence and absence of DPDPE = 5.73 \pm 0.31 and 5.81 \pm 0.11, $P = 0.82$, unpaired t test; five tissues each from four and five pigs, respectively).

Effects of Selective Opioid Antagonists on Agonist Activity. The nonselective OR antagonist naloxone slightly but significantly reduced the potency of DPDPE in suppressing neurogenic ion transport (Fig. 2; Table 3). The OR(s) modulating neurogenic ion transport was further characterized through a determination of the potencies of δ - and μ -OR antagonists in inhibiting agonist actions. None of the antagonists tested altered baseline or EFS-induced I_{sc} when administered before agonists. BNTX, an antagonist selective for the putative δ_1 -OR (Portoghese et al., 1992), significantly reduced the inhibitory potencies of DPDPE (Fig. 2), DAMGO (Fig. 3), and morphine (Fig. 4) with respective K_e values of 8.0, 6.9, and 12.8 nM (Table 3). BSINTX, another selective δ_1 -OR antagonist (Ohkawa et al., 1997), reduced the potency of DPDPE albeit with a higher K_e value (Table 3).

NTB, an antagonist at putative δ_2 -ORs (Takemori and Portoghese, 1992), was also less potent than BNTX (Table 3). It reduced DPDPE potency with a K_e values of 32.3 nM, but had no significant effect on DAMGO potency (Figs. 2 and 3; DAMGO pIC₅₀ in absence and presence of 100 nM NTB =

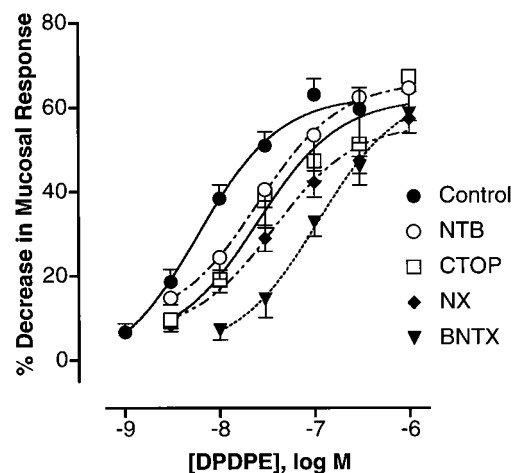


Fig. 2. Antagonism of the inhibitory action of the putative δ -opioid agonist DPDPE by 100 nM NTB, CTOP, naloxone (NX), or BNTX. Data represent the mean \pm S.E.M. of responses measured in 4 to 15 tissues from 3 to 15 pigs.

8.00 \pm 0.07 and 7.33 \pm 0.22; $P > 0.05$, Dunnett's t test, 11 and 5 tissues from 11 and 4 pigs, respectively). At a serosal concentration of 100 nM, NTB produced a significant 3.5-fold increase in morphine potency (Fig. 4; morphine pIC₅₀ in

TABLE 3
Potency of selective opioid receptor antagonists

Antagonist ^a	Opioid Receptor Selectivity	Agonist	pIC ₅₀	Fold-Decrease in Agonist Potency	Antagonist K _e	n Tissues (from N Pigs)
			<i>M</i>		<i>nM</i>	
Naloxone	Nonselective	DPDPE	7.55 ± 0.17*	3.6	38.0	4 (3)
BNTX	δ ₁	DPDPE	6.98 ± 0.10**	13.5	8.0	5 (4)
		DAMGO	6.81 ± 0.29**	15.5	6.9	5 (4)
		Morphine	4.92 ± 0.20**	7.8	12.8	5 (4)
		DPDPE	7.54 ± 0.13*	3.7	37.0	5 (4)
BSINTX	δ ₁	DPDPE	7.50 ± 0.18*	4.1	32.3	4 (4)
NTB	δ ₂	DPDPE	7.40 ± 0.08*	5.1	24.4	3 (3)
TIPP	δ	DPDPE	7.58 ± 0.15*	3.4	41.7	4 (4)
CTOP	μ	DPDPE				

* $P < 0.05$ and ** $P < 0.01$ versus agonist mean pIC₅₀ values in absence of antagonists, Dunnett's test.

^a Serosal bath concentration of 100 nM.

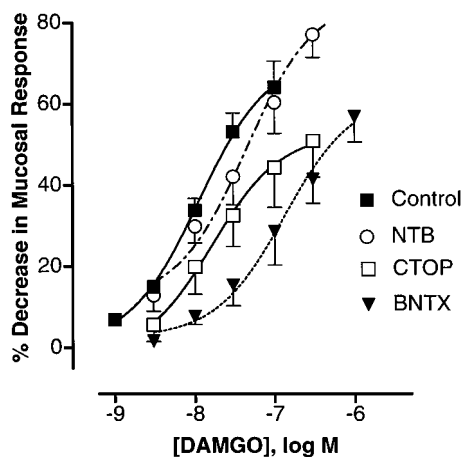


Fig. 3. Antagonism of the inhibitory action of the μ -opioid agonist DAMGO by 100 nM BNTX. Pretreatment of tissues with either NTB or CTOP did not significantly alter DAMGO potency (see text). Data represent the mean \pm S.E.M of responses measured in 4 to 11 tissues from 3 to 11 pigs.

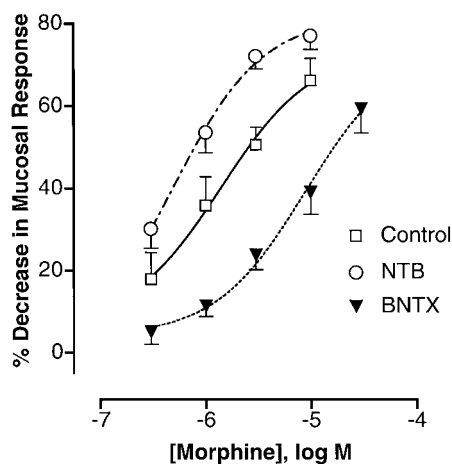


Fig. 4. Effects of δ -opioid antagonists on morphine action. The inhibitory potency of morphine was significantly reduced by 100 nM BNTX, but significantly increased by NTB. Data represent the mean \pm S.E.M. of responses measured in three to five tissues from three to five pigs.

presence and absence of NTB = 6.35 ± 0.09 and 5.81 ± 0.11 , $P < 0.05$, Dunnett's t test; four and five tissues from four and five pigs, respectively).

DPDPE potency was also decreased 5-fold by 100 nM TIPP (Schiller et al., 1999), a δ -OR antagonist that does not distinguish between the putative δ -OR subtypes. The antagonist

had no significant effect on DAMGO potency (DAMGO pIC₅₀ in presence of TIPP = 8.05 ± 0.16 , $P > 0.05$, Dunnett's t test; three tissues from three pigs). At a serosal concentration of 100 nM, CTOP, an antagonist selective for μ -OR (Kramer et al., 1989), did not significantly alter the potency of DAMGO (Fig. 3; DAMGO pIC₅₀ in presence of CTOP = 8.03 ± 0.44 , $P > 0.05$, Dunnett's t test; five tissues from four pigs). However, it produced a small, but significant reduction in DPDPE potency (Fig. 2; Table 3).

Expression of Opioid Receptor-Like Immunoreactivities in Submucosal Neurons. Using a primary antibody directed against an identical peptide sequence in the predicted second extracellular loop of mouse and porcine δ -OR (Arvidsson et al., 1995; Brown et al., 1998), δ -OR-like immunoreactivity was observed in neurons contained within the inner submucosal ganglia and in nerve fibers of the lamina propria (Fig. 5). No δ -OR-like immunoreactivity was observed in non-neuronal cells within the intestinal mucosa and submucosa. Anti- μ - or κ -OR antisera directed toward N-terminal peptide sequences in these receptors failed to detect μ -OR (Fig. 5) or κ -OR (data not shown) immunoreactivity in the mucosa or submucosa. However, μ -OR immunoreactivity was apparent in transverse sections of the porcine hypothalamus and in the myenteric plexus of guinea pig ileum (Fig. 6).

Discussion

Mucosa-submucosa preparations from the porcine ileum responded to ETS with transient increases in I_{sc} . In porcine distal jejunal mucosa, ETS similarly elevates I_{sc} , an effect associated with increased electrogenic anion secretion (Hildebrand and Brown, 1990). In both locations, ETS actions are mediated by submucosal neurons because they were inhibited after neural conduction blockade or ganglionic blockade. The precise chemical coding of neural circuits mediating the ion transport alterations evoked by ETS and inflammatory mediators in porcine jejunum and ileum remains to be elucidated. After serosal administration, OR agonists inhibited ETS-induced I_{sc} elevations in a concentration-dependent manner. Peptidic δ - and μ -OR agonists were more potent than the nonpeptidic δ -OR agonist SNC80, the μ -OR agonists morphine and loperamide, or the κ -OR agonists U-50,488H and U-69,593. In murine jejunum and porcine distal jejunum, DPDPE was respectively 353- and 25-fold more potent than U-50,488H in suppressing ETS-evoked I_{sc} elevations (Sheldon et al., 1990; Quito and Brown, 1991).

Indeed, U-50,488H and U-69,593 bind to δ -ORs in the concentrations at which they were effective in the porcine ileum (Goldstein and Naidu, 1989). In mouse jejunum, U-50,488H action was not mediated by ORs because it could not be inhibited by naloxone, ICI-174,864, or the selective κ -OR antagonist norbinaltorphimine (Sheldon et al., 1990).

The wide difference in potency between SNC80 and DPDPE or other peptidic δ -OR agonists was unexpected, because the potencies and efficacies of SNC80 and DPDPE are similar at cloned δ -ORs (Quock et al., 1999). However, SNC80 may act at different sites within the δ -OR than those recognizing enkephalin-based agonists. SNC80 and DPDPE differ in their abilities to down-regulate a mutant δ -OR possessing a deleted C terminus (Okura et al., 2000). Moreover, the binding of diethylbenzamide-derived δ -OR agonists such as SNC80 but not that of enkephalin-derived δ -OR agonists depends on a Trp²⁸⁴ residue in the third extracellular loop of the cloned δ -OR (Valiquette et al., 1996). The nucleotide sequence of the cloned porcine δ -OR is 93% identical to that of human δ -OR, and the peptide sequence encoding this loop, including Trp²⁸⁴, is completely conserved in cloned porcine δ -OR. Thus, the observed difference in agonist potencies is not due to a species-related difference in this ligand binding domain (Brown et al., 1998).

The potencies of the selective μ -OR agonists DAMGO, PL-017, and endomorphin I in inhibiting neurogenic ion transport were similar to those of the peptidic δ -OR agonists. A similarly narrow separation in the potencies of DAMGO and DPDPE for inhibiting neurogenic ion transport was reported in the porcine distal jejunal mucosa (Quito and Brown, 1991). In contrast, DPDPE was 41-fold more potent than DAMGO in suppressing neurogenic secretion in murine jejunal mucosa (Sheldon et al., 1990). DPDPE affinity at the cloned δ -OR is several orders of magnitude higher than that of DAMGO or morphine (Knapp et al., 1995). Although DAMGO may act at μ -ORs in the porcine ileal mucosa-submucosa, it was 155- and 309-fold more potent than morphine and loperamide, respectively. At cloned μ -ORs, μ -OR subtypes, or splice variants, however, there is little separation between the affinities of DAMGO and morphine (Pick et al., 1991; Knapp et al., 1995; Pan et al., 1999). The agonist potency differences observed in porcine ileum may be attributable to differences in drug efficacy or in agonist-induced receptor regulation. Indeed, unlike DAMGO, morphine does not induce μ -OR internalization (Sternini et al., 2000). Recently, a mutant μ -OR in which Trp³¹⁸ was replaced by a lysine residue exhibited similar affinities for DPDPE and DAMGO and lower affinities for both SNC80 and morphine (Bonner et al., 2000). Mutations of the Trp³¹⁸ residue probably remove steric hindrance and permit greater access to binding by opioid ligands (Metzger et al., 2001). When this barrier is removed, normally selective ligands lose their selectivity. However, the Trp³¹⁸ residue is conserved in the porcine μ -OR sequence (Pampusch et al., 1998).

The effects of DPDPE were reduced significantly by the nonselective OR antagonist naloxone with a K_e value suggestive of drug interactions with the δ -OR. Further experiments with δ - and μ -OR antagonists were performed to test the hypothesis that a common OR receptor mediates the actions of peptidic OR agonists in the porcine ileal mucosa-submucosa. DPDPE and DAMGO potencies were reduced by a similar magnitude in tissues pretreated with 100 nM BNTX, an

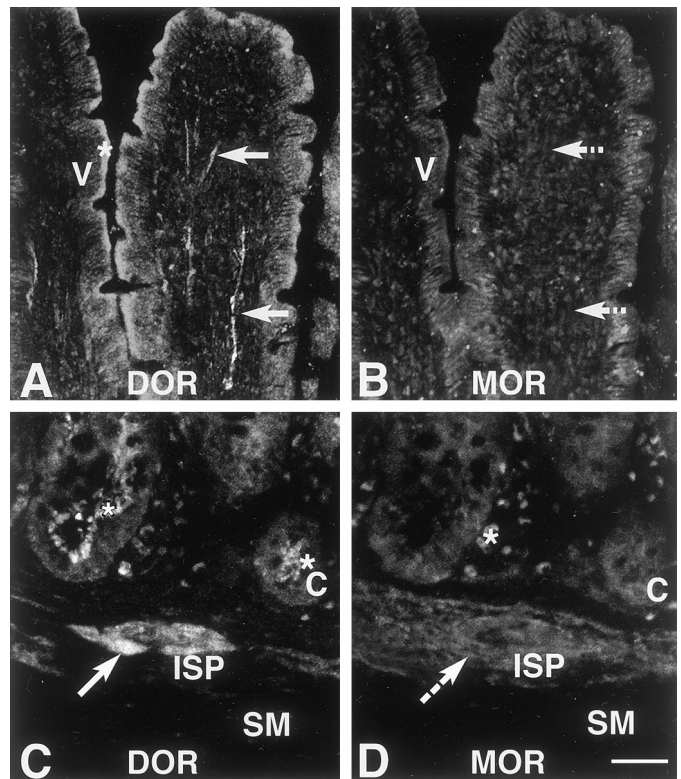


Fig. 5. Digitized confocal micrographs of representative longitudinal sections of muscle-stripped mucosa-submucosa from porcine ileum demonstrating the neuronal localization of immunoreactivity to the δ -OR and lack of immunoreactivity to the μ -OR. A, immunoreactivity to δ -OR in varicose nerve fibers (arrows) present in the lamina propria within intestinal villi (V). B, identical tissue section as in A, showing the absence of specific μ -OR immunoreactivity (broken arrows). C, immunoreactivity to δ -OR in neuronal perikarya (arrow) within the inner submucosal plexus (ISP) lying below an intestinal crypt (c). D, identical tissue section as in C, showing the absence of specific μ -OR immunoreactivity (broken arrow). Asterisks indicate nonspecific immunoreactivity that remained in preabsorption control experiments. The tissue sections are representative of observations made in ileal mucosa-submucosa sheets from five pigs. Scale bar, 100 μ m.

antagonist selective for the putative δ_1 -OR. In agreement with a previous study (Ohkawa et al., 1997), BNTX was more potent than BSINTX, another selective δ_1 -OR antagonist in reducing DPDPE potency. It was also 3- to 4-fold more potent than the putative δ_2 -OR-selective antagonist NTB. The inhibitory potency of morphine was also reduced by BNTX, but not by NTB. In the ileal mucosa TIPP or CTOP, antagonists selective for δ - and μ -ORs, respectively, produced slight, but

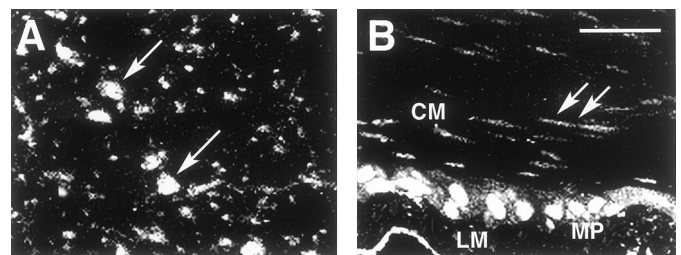


Fig. 6. Digitized confocal micrographs of μ -opioid receptor-like immunoreactivity on neurons and nerve fibers in the porcine hypothalamus (arrows indicate representative neurons) (A) and myenteric plexus (MP) and smooth muscle of guinea pig ileum (double arrows indicate immunoreactive nerve fibers in circular muscle) (B). Micrographs represent transverse sections of hypothalamus and ileum. CM, circular muscle; LM, longitudinal muscle. Scale bar, 120 μ m.

significant reductions in the potency of DPDPE, but not of DAMGO. Thus, the effects of δ - and μ -OR agonists are mediated by a receptor which, based on experiments with OR antagonists, appears to have the pharmacological characteristics of a BNTX-preferring δ_1 -OR. Because morphine exhibited a low potency in inhibiting neurogenic ion transport and its effects remained unaltered in tissues pretreated with DPDPE, it is unlikely that this OR represents a " $\delta_{\text{complexed}}$ " receptor (Rothman et al., 1988). DPDPE and DAMGO bind with nearly equal affinities to a recently described heterodimeric μ/δ -OR binding site (George et al., 2000; Gomes et al., 2000). Because its pharmacological characteristics have not been examined in sufficient detail with some of the subtype-selective OR ligands used in the present study, it is not yet possible to relate it to the OR in the ileal submucosa. As might be predicted from previous studies of μ/δ -OR heterodimers, the δ -OR antagonist NTB appears to potentiate the actions of morphine. However, the findings that the selective μ -OR antagonist CTOP decreases DPDPE potency and does not affect DAMGO activity in this preparation argues against this possibility.

The results of immunohistochemical experiments support the functional data indicating that δ -ORs, but not μ - and κ -ORs are expressed in the mucosa-submucosa preparation from porcine ileum. An antibody raised against an identical peptide sequence present in the second extracellular loops of murine and porcine δ -ORs (Table 1) detected specific δ -OR-like immunoreactivity in submucosal neurons and nerve fibers within the lamina propria. This distribution pattern is similar to that previously reported in the porcine small intestine with an antibody raised against the N terminus of the cloned murine δ -OR (Brown et al., 1998). No δ -OR-like immunoreactivity was localized in enterocytes. The apparent expression of δ -OR binding sites in rat and guinea pig enterocytes may reflect species differences in the cellular expression of this receptor (Lang et al., 1996; Nano et al., 2000). Because the pharmacological characteristics of the submucosal OR scrutinized in this study appear to be very different from those of the cloned δ -OR, it is interesting that antisera raised against peptide sequences within the cloned δ -OR permitted detection of δ -OR-like immunoreactivity in the submucosa. It is possible that epitopes present in the cloned δ -OR are also present in the submucosal OR, which may represent a δ/μ -OR heterodimer. On the other hand, different δ -OR subtypes might coexist in the intestine as they appear to do in central nervous system, and the submucosal OR mediating mucosal function may not have been detected by the immunohistochemical procedure. No specific immunoreactivity was observed to either κ - or μ -ORs in intestinal mucosa or submucosa. Because κ -OR immunoreactivity is observed in myenteric neurons and fibers of the porcine ileum with the same antibody (Poonyachoti et al., 2001), it appears that κ -ORs are not expressed in the intestinal mucosa, which is consistent with the functional data. The anti- μ -OR antibody used in these studies was raised against an N-terminal epitope in human μ -OR and may not have detected the homologous peptide sequence in porcine μ -OR, which displays 70% sequence identity with human μ -OR (Table 1). However, it recognized specific μ -OR-like immunoreactivity in sections of the porcine hypothalamus and guinea pig ileum. It should detect μ -OR splice variants as well, because exon 1 of the μ -OR gene encodes the N terminus of

μ -OR, and any potential splice junctions occur downstream from this exon (Pan et al., 1999). The results may indicate that the μ -OR is not present in the porcine intestinal mucosa or that it is in a form (such as a receptor heterodimer) in which the epitope is not accessible to antibody binding.

In sum, we have identified an OR in the submucosa of the porcine small intestine that mediates the actions of peptidic OR agonists on neurogenic ion transport. Although it appears to recognize both δ - and μ -OR ligands, it possesses pharmacological characteristics that are not typical of the presently cloned δ - or μ -ORs, but may be representative of a novel OR subtype or as in the case of the μ -OR (Pan et al., 1999), a δ -OR splice variant. Because it modulates neurogenic ion transport in the ileum evoked by ETS and inflammatory mediators, this receptor probably participates in non-specific intestinal host defense (Green et al., 2000; Poonyachoti and Brown, 2001). It may also be involved in preserving mucosal function under ischemic conditions (Mayfield and D'Alecy, 1994). This novel receptor deserves additional pharmacological and molecular characterization in future investigations.

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