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Characterization of specific δ-opioid binding sites in the distal small intestine of swine

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Abstract

Delta-opioid receptors modulate neurogenic smooth muscle contractions and mucosal ion transport in the porcine distal small intestine. We compared specific δ-opioid binding sites in neuronal membranes isolated from the inner submucosal plexus and myenteric plexus of this gut region. In both preparations, the δ-opioid receptor antagonist naltrindole displaced [^{3}H]diprenorphine from two binding sites. [^{3}H]Naltrindole bound specifically to a single high-affinity site that was displaced by δ-opioid receptor ligands with the rank order of affinity: naltriben >7-benzylidenenaltrexone>deltorphin II>[D-Pen^2, D-Pen^5] enkephalin. Relative decreases in δ-opioid receptor agonist affinity in the presence of Na^+ and other ions differed in submucosal and myenteric membranes. The κ-opioid receptor antagonist norbinaltorphimine displaced [^{3}H]naltrindole binding in myenteric membranes (K_i = 7.2 nM). Delta-opioid receptors in submucosal and myenteric plexuses of porcine ileum appear to differ in some respects.

Keywords: Opioid receptor; Enteric nervous system; Naltrindole; Diprenorphine (Pig)

1. Introduction

The clinically important antisecretory and antimotility actions of opioid antidiarrheal drugs are mediated in part by μ-, δ- and κ-opioid receptors located within the enteric nervous system (DeLuca and Coupar, 1996). In the porcine small intestine, the δ-opioid receptor appears to be the predominant opioid receptor type expressed on enteric neurons. This has been determined in functional assays of the suppressive effects of opioids on neurogenic smooth muscle contractions and mucosal ion transport (Poonyachoti et al., 2001a,b) and by radioligand binding to membrane preparations containing the myenteric plexus or the submucosal ganglionated plexuses (Townsend and Brown, 2002).

Evidence for distinct δ-opioid receptor subtypes in the central nervous system has been obtained in studies employing relatively complex in vivo models (Zaki et al., 1996). Putative δ_1-opioid receptors are generally defined as those exhibiting high affinity for the δ-agonists [D-Pen^2, D-Pen^5] enkephalin (DPDPE) and 7-benzylidenenaltrexone (BNTX); in comparison, δ_2-type receptors prefer deltorphin II and naltriben. Previous functional studies suggest that the pharmacological characteristics of δ-opioid receptors in the myenteric plexus differ from those in the inner submucosal plexus of the porcine ileum (Poonyachoti et al., 2001a,b). In the present investigation, we tested the hypothesis that a heterogeneous population of δ-opioid receptors exists in the porcine enteric nervous system through an examination of the ligand binding characteristics of myenteric and inner submucosal δ-opioid receptors with highly selective opioid agonists and antagonists. Furthermore, ligand binding in enteric neuronal membranes incubated in a conventional, Na^+-free Tris buffer was compared to that occurring in a physiologically relevant medium, i.e. Krebs–HEPES buffer.

2. Materials and methods

2.1. Tissue isolation

A segment of distal small intestine was obtained from each of 30 weaned, outbred Yorkshire pigs of each sex (6–10 weeks of age; 10–18 kg body weight). Animals were not fasted before sacrifice. They were sedated with an intramuscular injection of tiletamine hydrochloride–zolazepam

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(8 mg/kg, Fort Dodge Laboratories, Fort Dodge, IA), in combination with xylazine (3 mg/kg, Phoenix Pharmaceuticals, St. Joseph, MO). The animals were subsequently euthanized by barbiturate overdose in accordance with approved University of Minnesota Institutional Animal Care and Use Committee protocols. A midline laparotomy was performed and an intestinal segment, including that portion attached to the ileocecal ligament, was resected extending approximately 1.5 m orad from the ileocecal junction. Subsequent tissue dissections were performed at 4 °C.

2.2. Neuronal membrane isolation

Enteric neuronal membranes were isolated as described previously (Townsend and Brown, 2002) using either Na^+-free Tris (50 mM Tris) buffer or Krebs–HEPES buffer (composition in mM: NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgCl_2$, 1.2 and HEPES, 25). The pH of both buffers was corrected to 7.4 by the addition of a 6 M HCl solution. The muscularis externa containing the myenteric and deep muscular plexuses was carefully separated from the underlying submucosa and mucosa. The mucosa was removed at the level of the muscularis mucosa, yielding a sheet of connective tissue containing the inner submucosal (Meissner) plexus. Portions of the outer submucosal (Schabadasch) plexus were present in both myenteric and submucosal preparations. Both tissue preparations were diced into small pieces and homogenized using a Polytron (PT 3000; Brinkmann, Westbury, NY). Enteric neuronal membranes were enriched from these tissues through a series of centrifugation steps. Following isolation, P2 membrane fractions were stored at −70 °C until binding assays were performed. Neuronal enrichment and membrane protein integrity of the P2 fraction were assessed by examining the tetrodotoxin (1 μM)-displaceable binding of [^{3}H]saxitoxin, as previously described (Townsend and Brown, 2002). Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

2.3. Chemicals and drugs

[^{3}H]Naltrindole (35 Ci/mmol) and [^{3}H]diprenorphine ((5α,7α)-17-(cyclopropylmethyl)-4,5-epoxy-18,19-dihydro-3-hydroxy-6-methoxy-α,α-dimethyl-6,14-ethenomorphinan-7-methanol; 70 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). All radioligands were diluted to the desired concentration in 5 mM HCl and stored at −20 °C until use. Naloxone was obtained from Sigma (St. Louis, MO). [D-Pen^2, D-Pen^5]enkephalin (DPDPE), [D-Ala^2, Glu^4]deltorphin II, and [D-Ala^2, *N*-methyl-Phe^4, Gly^5-ol]enkephalin (DAMGO) were obtained from Bachem (Torrance, CA). Naltriben, 7-benzylidenenaltrexone (BNTX), and norbinaltorphimine were generously provided by Dr. Philip S. Portoghese (Department of Medicinal Chemistry, University of Minnesota). All other reagents were obtained from Fisher Scientific (Chicago, IL).

2.4. Radioligand binding experiments

Isolated neuronal membranes were thawed on ice the day of the experiment and diluted, with either the Tris or Krebs–HEPES buffers described above, to a final concentration of 500 μg/ml. The actual protein concentration was determined from an aliquot of the diluted membrane used in the assay. Displacement assays characterized the binding properties of unlabeled ligands by the displacement of the nonselective opioid ligand [^{3}H]diprenorphine (1 nM) or the δ-opioid receptor ligand [^{3}H]naltrindole (0.1 nM) from specific binding sites. Saturation analyses of specific δ-opioid binding sites were performed using [^{3}H]naltrindole (0.003–0.3 nM); nonspecific binding was determined in the presence of 1 μM unlabeled naloxone. Following a 60-min incubation at room temperature with shaking, the membranes were subjected to rapid filtration of membranes using a Brandel (Gaithersburg, MD) 24-sample cell harvester; membranes were washed with 4 ml of cold buffer to remove any unbound ligand. Filters containing membranes were submerged in scintillation fluid for approximately 12

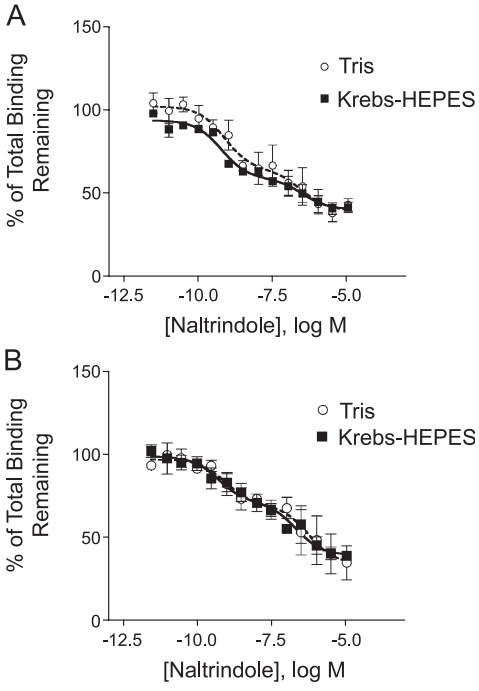

Fig. 1. Displacement of 1 nM [^{3}H]diprenorphine from inner submucosal (A) and myenteric (B) neuronal membranes by the δ-opioid receptor antagonist naltrindole. Assays were performed on membranes incubated in either Tris (○) or Krebs–HEPES (■) buffers as described in Materials and methods. The ordinate indicates the percentage of the total [^{3}H]diprenorphine binding remaining, and the abscissa indicates the $\log_{10}$ molar concentration of naltrindole. Thick solid lines and dashed lines indicate the results of the displacement assays performed in membranes incubated in Krebs–HEPES and Tris buffer, respectively, and subjected to nonlinear regression analysis. The integrated results of the regression analyses are presented in Table 1. Each point represents the mean ± S.E. % of total [^{3}H]diprenorphine binding determined in three to four replications with neuronal membranes from three to four pigs.

Table 1
Affinity constants and displacing ability of unlabeled naltrindole at high ($K_{i(H)}$) and low ($K_{i(L)}$) affinity [^{3}H]diprenorphine binding sites in inner submucosal plexus or myenteric plexus membranes from porcine ileum

Incubation buffer	Inner submucosal plexus				Myenteric plexus			
	$K_{i(H)}$ (nM)	$K_{i(L)}$ (nM)	%Maximum displacement of total radioligand binding	%Maximum displacement from high-affinity radioligand binding site	$K_{i(H)}$ (nM)	$K_{i(L)}$ (nM)	%Maximum displacement of total radioligand binding	%Maximum displacement from high-affinity radioligand binding site
Tris	0.136 (0.029–0.645)	50.7 (3.1–830.7)	60.1 (48.6–71.6)	60.7 (38.9–82.6)	0.160 (0.019–1.362)	94.7 (12.4–723.0)	65.4 (51.0–79.8)	43.7 (24.0–63.4)
Krebs–HEPES	0.219 (0.090–0.531)	116 (18–7480)	59.5 (53.3–65.7)	65.3 (52.8–77.8)	0.189 (0.033–1.076)	73.3 (14.7–366.5)	60.2 (51.9–68.6)	46.6 (29.2–63.9)

Results were obtained from nonlinear regression of [^{3}H]diprenorphine displacement assays. Maximal displacement of [^{3}H]diprenorphine and relative amount of high affinity [^{3}H]diprenorphine binding are expressed respectively as a percentage of total [^{3}H]diprenorphine binding and as a percentage of maximum displacement. All data are expressed as mean values with 95% confidence intervals in parentheses. The results are based on experiments with three to four replications using neuronal membranes isolated from three to four pigs.

h before determination of radioligand concentration by liquid scintillometry.

2.5. Data analysis

In saturation analyses, specific binding was determined by calculating the difference between [^{3}H]naltrindole binding in the presence and absence of 1 μM naloxone. The resulting data were averaged at each concentration and analyzed by nonlinear regression. Similarly, data from [^{3}H]diprenorphine and [^{3}H]naltrindole displacement experiments were averaged and analyzed using nonlinear regression; in these experiments, affinity constants (K_i) values were calculated by the method of Cheng and Prusoff (1973). In all cases, a single binding site model was chosen unless a two-site model gave a significantly ($P < 0.05$) better fit by F-test. Nonlinear regres-

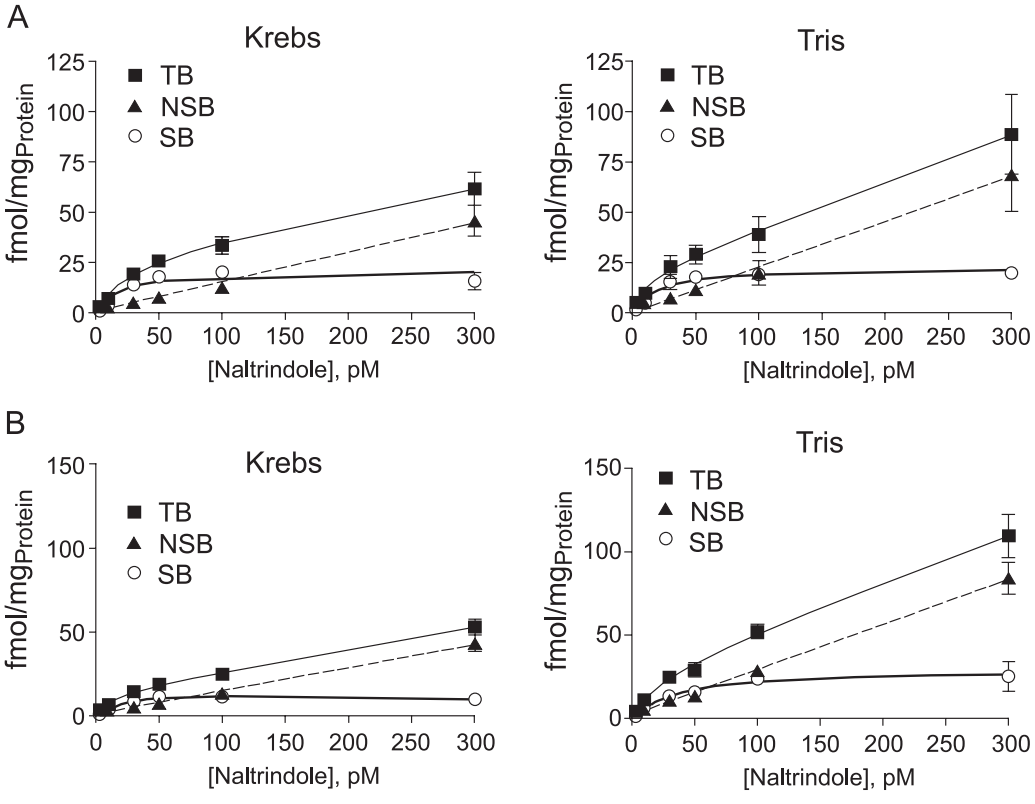

Fig. 2. Saturation analyses of [^{3}H]naltrindole binding to specific, high-affinity sites in neuronal membranes from the inner submucosal plexus (A) or myenteric plexus (B) of porcine distal small intestine. Membranes were bathed in either Krebs–HEPES or Tris buffer as described in Materials and methods. Specific binding (SB; ○), nonspecific binding (NSB; ▲) and total binding (TB; ■) are shown. Ordinate indicates binding site density in fmol/mg protein, and abscissa indicates radioligand concentration. The thick line represents the results of the nonlinear regression of the SB data. The thin solid and dashed lines represent the best fit of nonlinear and linear functions to TB and NSB data, respectively. The integrated results of the regression analyses are presented in Table 2. Each point represents the mean ± S.E. of [^{3}H]naltrindole binding determined in 4–11 replications with neuronal membranes from three to seven pigs.

Table 2
Summary of K_d and B_{max} values obtained from saturation analyses of specific [^{3}H]naltrindole binding to submucosal plexus and myenteric plexus membranes

Buffer	Inner submucosal plexus		Myenteric plexus	
	K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)
Tris	20.9 (3.5–38.4)	22.9 (17.7–28.0)	38.7 (2.1–75.3)	30.0 (20.3–39.6)
Krebs–HEPES	19.2 (0.1–38.0)	21.2 (26.9–15.6)	15.0 (5.0–24.9)	12.2 (10.2–14.2)

All data are expressed as mean values with 95% confidence intervals in parentheses. They are based on experiments with 4–11 replications using membranes isolated from three to seven pigs.

sions were performed using the Prism statistical software package (ver. 3.0c; GraphPad, San Diego, CA).

3. Results

3.1. Binding of naltrindole to enteric neuronal membranes

The nonselective opioid receptor antagonist [^{3}H]diprenorphine was displaced by the selective δ-opioid receptor antagonist naltrindole from two specific binding sites in neuronal membranes isolated from either the myenteric plexus or inner submucosal plexus (Fig. 1; Table 1). In both preparations, [^{3}H]naltrindole bound specifically to a single population of binding sites with subnanomolar affinity (Fig. 2; Table 2). Its affinity for these sites was considerably higher than that of the naltrindole-displaceable, higher affinity binding site for [^{3}H]diprenorphine and did not differ significantly in membranes bathed in Na^+-free Tris buffer or Krebs–HEPES buffer. However, the density (B_{max}) of [^{3}H]naltrindole binding sites was relatively low in myenteric membranes incubated in Krebs–HEPES buffer (Table 2).

3.2. Displacement of [^{3}H]naltrindole from submucosal neuronal membranes by opioid receptor ligands

The displacement of 0.1 nM [^{3}H]naltrindole by unlabeled ligands was used to examine further the pharmacological characteristics of high affinity [^{3}H]naltrindole binding sites in submucosal membranes. At this radioligand concentration, the high affinity site exhibited complete saturation with negligible radioligand binding at the lower affinity site. In submucosal membranes, the δ-opioid receptor antagonists naltriben and BNTX displaced [^{3}H]naltrindole from its high-affinity binding site with respective K_i's of 0.05 and 2.22 nM (Table 3). Although the affinities of these ligands did not change significantly in the presence of physiological concentrations of ions, the relative maximum displacement of total [^{3}H]naltrindole binding by BNTX was greater in membranes incubated in Krebs–HEPES buffer than in Tris buffer (Table 3).

The δ-opioid receptor agonists DPDPE and deltorphin II maximally displaced [^{3}H]naltrindole binding at a magnitude similar to the antagonists. In comparison with the four δ-opioid receptor ligands, the highly selective μ-opioid receptor agonist DAMGO manifested >270-fold lower affinity for the high-affinity [^{3}H]naltrindole binding site (Table 3). The affinities of DPDPE and deltorphin II decreased by a similar magnitude (19- and 24-fold, respectively) in submucosal membranes incubated in Krebs–HEPES buffer relative to their affinities determined in membranes incubated in Tris buffer (Table 3).

3.3. Displacement of [^{3}H]naltrindole from myenteric neuronal membranes by opioid receptor ligands

As in submucosal membranes, the affinities of naltriben and BNTX for high-affinity [^{3}H]naltrindole binding sites were not significantly different in myenteric membranes incubated in Tris or Krebs–HEPES buffers (Table 4). The affinities of DPDPE and deltorphin II in displacing high-affinity [^{3}H]naltrindole binding under Na^+-free conditions were decreased by 34- and 13-fold, respectively, in membranes incubated in Krebs–HEPES buffer (Table 4).

The κ-opioid receptor appears to be expressed in the porcine ileal myenteric plexus where it is colocalized with δ-opioid receptors in a subpopulation of enteric neurons, based on the results of previous functional, immunohistochemical and radioligand experiments (Poonyachoti et al., 2001a, 2002; Townsend and Brown, 2002). The highly selective κ-opioid receptor antagonist norbinaltorphimine displaced specific, high affinity [^{3}H]naltrindole binding

Table 3
Affinities and magnitudes of [^{3}H]naltrindole displacement from its high affinity site in submucosal neuronal membranes by opioid ligands

Ligand	Tris		Krebs–HEPES	
	K_i (nM)	%Displaced	K_i (nM)	%Displaced
BNTX	2.22 (0.97–5.13)	58.1 (50.7–65.6)	1.81 (1.04–3.15)	79.0 (72.3–85.7)
Naltriben	0.0509 (0.0208–0.1246)	67.5 (61.6–73.5)	0.193 (0.117–0.379)	73.2 (67.9–78.6)
DPDPE	2.94 (1.43–6.06)	48.3 (44.1–52.6)	56.4 (18.0–176)	59.8 (39.6–80.0)
Deltorphin II	1.76 (0.87–3.52)	52.0 (48.2–55.7)	42.7 (19.1–95.2)	62.6 (53.7–71.5)
DAMGO	808 (35–1870)	51.8 (41.4–62.2)	13,000 (1620–104,000)	70.3 (−2.8–143.1)

The results were obtained from nonlinear regression analysis of displacement assay data. All data are expressed as mean values with 95% confidence intervals in parentheses and are based on experiments with three to seven replications, using membranes isolated from three to five pigs.

Table 4
Affinities and magnitudes of [^{3}H]naltrindole displacement from its high affinity site in myenteric neuronal membranes by opioid ligands

Ligand	Tris		Krebs–HEPES	
	K_i (nM)	%Displaced	K_i (nM)	%Displaced
BNTX	1.53 (0.72–3.25)	64.4 (58.3–70.6)	2.86 (0.84–9.72)	49.7 (39.9–59.5)
Naltriben	0.117 (0.043–0.318)	37.6 (32.5–42.8)	0.196 (0.079–0.490)	48.9 (43.1–54.6)
DPDPE	1.24 (0.62–2.45)	52.0 (46.0–58.1)	42.2 (12.6–141.2)	45.0 (35.3–54.8)
Deltorphin II	1.69 (0.69–4.13)	58.7 (51.8–65.7)	21.8 (9.8–48.2)	47.9 (40.3–55.4)
Norbinaltorphimine	7.24 (2.07–25.27)	39.9 (31.8–48.1)	11.0 (3.9–30.9)	45.9 (36.4–55.5)

The results were obtained from nonlinear regression analysis of displacement assay data. All data are expressed as mean values with 95% confidence intervals in parentheses and are based on experiments with 3–10 replications, using membranes isolated from three to six pigs.

from myenteric membranes with K_i's of 7.2 and 11.0 nM in myenteric membranes incubated in Tris and Krebs–HEPES buffer, respectively (Table 4).

4. Discussion

The displacement of [^{3}H]diprenorphine by naltrindole from two binding sites in myenteric and inner submucosal membranes is indicative of heterogeneity in the population of opioid receptors present in the porcine enteric nervous system. Delta-opioid receptors appear to represent a major portion of this receptor population, especially in submucosal membranes, in which naltrindole displaced $\geq 60\%$ of specific [^{3}H]diprenorphine binding from a high-affinity site. In both myenteric and submucosal membranes, the equilibrium binding constants determined for naltrindole at the high-affinity [^{3}H]diprenorphine binding site are similar to those determined for naltrindole binding to recombinant δ-opioid receptors (Raynor et al., 1994). The lower affinity site for naltrindole matches the binding characteristics expected for binding to an opioid receptor other than the δ-type in enteric neural membranes; naltrindole has a K_d of 66 and 64 nM for κ- and μ-opioid receptors, respectively (Raynor et al., 1994).

At subnanomolar concentrations, [^{3}H]naltrindole bound to a single, specific binding site in myenteric and submucosal membranes. Its affinities at these sites were similar to those observed with native δ-opioid receptors in other tissue preparations as well as with cloned δ-opioid receptors in cell expression systems (Clark et al., 1997; Fang et al., 1994). A reduction in the density of [^{3}H]naltrindole binding sites was detected in myenteric membranes incubated in Krebs–HEPES buffer compared to those incubated in Na^+-free Tris. This phenomenon may result from intermolecular associations between non-integral membrane proteins with myenteric membranes occurring in the physiologically appropriate medium. The inclusion of additional centrifugation steps in the membrane isolation procedure eliminates this disparity in myenteric binding site densities determined in Tris and Krebs–HEPES buffers (D. Townsend and D.R. Brown, in press). The ligand recognition characteristics of high affinity [^{3}H]naltrindole binding sites in submucosal and myenteric membranes suggest that they are similar in their pharmacological properties to the cloned δ-opioid receptor. Delta-opioid receptors cloned from rodents exhibit a relative affinity order of naltriben>BNTX>deltorphin II>DPDPE both in the presence and absence of Na^+ and Mg^{2+} ions (Clark et al., 1997; Raynor et al., 1994). The high affinity [^{3}H]naltrindole binding site detected in submucosal and myenteric membranes exhibits an identical rank order of affinities for these displacing ligands. The selective μ-opioid receptor agonist DAMGO exhibited low affinity for the specific [^{3}H]naltrindole binding site in submucosal membranes, a result consistent with the identity of this binding site as a δ-opioid receptor. Myenteric, but not submucosal ganglia have previously been found to express κ-opioid receptors (Poonyachoti et al., 2001a; Townsend and Brown, 2002). Predictably, norbinaltorphimine displaced high-affinity [^{3}H]naltrindole binding in the presence of Tris or Krebs–HEPES buffer with respective K_i's of 7.2 and 11 nM in myenteric neural membranes. This κ-opioid receptor antagonist is reported to bind to κ-opioid receptors with a K_i values varying from 0.027 to 4.9 nM; these same studies indicate that norbinaltorphimine binds to the δ-opioid receptor with affinities ranging from 32 to 65 nM (Jordan and Devi, 1999; Raynor et al., 1994). The apparent high affinity of norbinaltorphimine for the [^{3}H]naltrindole binding site in myenteric membranes may suggest that this δ-opioid receptor binding site has been modified. One possible explanation for this altered binding profile may be a functional association between δ- and κ-opioid receptors expressed in the same neuron (Poonyachoti et al., 2001a; D. Townsend and D.R. Brown, in press). However, the affinity of norbinaltorphimine for δ-/κ-opioid receptor heterodimers expressed in cultured cells is even lower than that for δ-opioid receptors (Jordan and Devi, 1999).

Predictably, the affinities of the δ-opioid receptor agonists DPDPE and deltorphin II but not those of the antagonists BNTX and naltriben for the high-affinity [^{3}H]naltrindole binding site in submucosal and myenteric membranes were decreased in the presence of Na^+ and other ions at physiologically appropriate concentrations, due to the increased proportion of G-protein-uncoupled receptors for which agonists have lower affinity. In studies with the cloned δ-opioid receptor, the Na^+-dependent decrease in agonist affinity relative to agonist affinity under Na^+-free conditions is much greater (61- vs. 18-fold) for DPDPE than for deltorphin II (Clark et al., 1997). A similarly large decrease in the affinity

of DPDPE relative to that of deltorphin II was detected in myenteric membranes incubated in Krebs–HEPES buffer. In submucosal membranes, however, Na^+-induced reductions in DPDPE and deltorphin II affinities for the specific [^{3}H]naltrindole binding site were of a similar magnitude. This apparent difference in agonist binding characteristics might be ascribed to trivial differences in the experimental protocol. However, it is of interest in view of the seemingly atypical pharmacological characteristics of δ-opioid receptors mediating suppression of neurogenic ion transport in sheets of porcine ileal mucosa with attached submucosa (Poonyachoti et al., 2001b; D. Townsend and D.R. Brown, 2003, referring to the British Journal of Pharmacology paper).

Specific δ-opioid binding sites in the porcine enteric nervous system appear to exhibit ligand recognition characteristics that are similar to those previously reported for native and cloned δ-opioid receptors. However, some differences in [^{3}H]naltrindole binding sites present in submucosal and myenteric membranes are apparent which may be due to the unique pharmacological properties of δ-opioid receptors in these intestinal subregions (Poonyachoti et al., 2001a,b; Townsend and Brown, 2003). Additional studies of the differences between these two enteric opioid receptors may offer important insights into the biology of opioid receptors in the central nervous system.

Acknowledgements

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