Predominance of δ-Opioid-Binding Sites in the Porcine Enteric Nervous System

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ABSTRACT

The antidiarrheal and constipating actions of opioids are mediated in part by enteric neurons, which lie within the wall of the small intestine and colon, but the differential expression of specific, high-affinity opioid-binding sites in ganglionated plexuses within functionally distinct intestinal segments has not been examined. We determined the saturation binding characteristics under Na+-free conditions of the nonselective opioid receptor (OPR) ligand [3H]Naltrindole, [3H]DAMGO, and [3H]-Me-Phe4,Gly5-ol-17-(cyclo)propanecarboxylic acid (CPC) and the respective δ-, κ- and μ-OPR ligands [3H]naltrindole, [3H][D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO) in neuronal membranes isolated from myenteric and submucosal plexuses of porcine small intestine and colon. Naloxone-displaceable [3H]diprenorphine-binding sites (Kd values ranging from 0.2–0.5 nM and Bmax = 50–95 fmol/mg of protein) were found in both subregions from all gut segments examined. High-affinity [3H]naltrindole sites (Kd = 60–140 pmol) were at highest densities (approximately 60 fmol/mg of protein) in submucosal plexus of ileum and distal colon and were at lowest densities (8–9 fmol/mg of protein) in the submucosal plexuses of cecum and distal colon. [3H]U-69,593 sites (Kd = 0.3–4 nM) were present only in the myenteric plexuses of all segments examined, with highest densities in cecum and proximal colon (44–47 fmol/mg of protein). [3H]DAMGO-binding sites were expressed at relatively low densities in the enteric plexuses of all gut regions. These results indicate that δ-OPRs predominate in the porcine enteric nervous system with a more circumscribed expression of κ- and μ-OPRs.

Opium has been employed in the treatment of diarrheal disease for centuries. Indeed, the opiate alkaloids morphine and codeine and the peripherally selective opioid loperamide continue to be among the most effective anti diarrheal agents in clinical use (Schiller, 1995). In addition, the endopeptidase inhibitor racemadotril has been found to effectively arrest acute diarrhea in children and adults by naloxone-sensitive mechanism (Matheson and Noble, 2000). The gastroenterologic use of opioids has recently been extended to the antidiarrheal and constipating actions of opioids by opioid agonists is associated with neuronal hyperpolarization or reduced neurotransmitter release due to the G protein-coupled activation of K+ channels or inhibition of N-type Ca2+ channel gating in myenteric and submucosal neurons (Cherubini and North, 1985; Mihara and North, 1986; Surprenant et al., 1990). Both inhibitory and excitatory enteric neurons may express OPRs (De Luca and Coupar, 1996). Immune-reactivities for at least some of the cognate endogenous ligands for these receptors, dynorphin and the enkephalins, are expressed in neurons and nerve fibers in either the myenteric or submucosal plexuses along the length of the intestinal tract (Kromer, 1990). Despite the neurobiological and clinical significance of intestinal OPRs, there have been no studies designed to examine the regional distribution, densities, and pharmacological characteristics of OPRs in structurally and functionally distinct intestinal segments. In the rat intestine, for example, OPR mRNA expression has been determined by ribonuclease protection assays (Fickel et al., 1997) or reverse transcriptase-polymerase chain reactions (Wittert et al., 1996). The neuronal localization of OPR proteins has been examined also by immunohistochemical approaches (Bagnol et al.,

ABBREVIATIONS: OPR, opioid receptor; DAMGO, [D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin; diprenorphine, (5α,7α)-17-(cyclopropylmethyl)-4,5-epoxy-18,19-dihydro-3-hydroxy-6-methoxy-α, α-dimethyl-6,14-ethenomorphinan-7-methanol; NTI, naltindole; STX, saxitoxin; U-69,593, (+)-5α,7α, 8β-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide; CI, confidence interval.
However, these studies do not provide information about the pharmacological characteristics and functional identity of enteric OPRs. Quantitative receptor autoradiography has been used in attempts to ascertain both the distribution and ligand specificity of opioid-binding sites in the rodent and porcine intestinal tract, but this approach has been limited by poor cellular resolution of binding sites and inadequate quantitation of OPR densities within the intestinal wall (Nishimura et al., 1986; James et al., 1987; Quito et al., 1991).

Radioligand binding to sites in cell membrane homogenates remains the most direct and quantitative means for measuring the pharmacological characteristics and densities of OPRs in tissues. In the intestine, especially in neuronally enriched fractions, ligand-binding assays to OPRs require significant amounts of starting materials. This problem has been overcome by reducing regional specificity (Creese and Snyder, 1975), pooling membranes from several animals (Monferini et al., 1981), or selecting large animal species as tissue donors (Allescher et al., 1989). The latter approach has been limited thus far to a characterization of OPRs in the canine ileum through the displacement of the nonselective OPR ligand \[^{3}H\](5α,7α-17-(cyclopropylmethyl)-4,5-epoxy-18,19-dihydro-3-hydroxy-6-methoxy-α,α-dimethyl-14-ethylmorphinan-7-methanol) (diprenorphine) by type-selective OPR ligands (Ahmad et al., 1989; Allescher et al., 1989).

In this study, we addressed the hypothesis that multiple OPRs are expressed in the submucosal and myenteric plexuses within the small intestine and colon. The pig was used as a tissue donor because the porcine intestine is considered to be a homolog of the human intestine (Almond, 1996). In the porcine small intestine, δ-OPR immunoreactivity has been localized in myenteric and submucosal neurons, and opioid agonists inhibit neurogenic contractions of intestinal smooth muscle and mucosal ion transport (Quito and Brown, 1991; Brown et al., 1998; Poonyachoti et al., 2001a,b). Saturation analyses of high-affinity, specific opioid binding were performed using radiolabeled ligands with high selectivity for each OPR type, with neuronal membranes isolated from both the myenteric and submucosal plexuses along the length of the porcine intestine.

### Materials and Methods

**Radioligands, Drugs, and Reagents.** The radioligands \[^{3}H\]saxitoxin (STX; 14.9 Ci/mmol) and \[^{3}H\]diprenorphine (70 Ci/mmol) were obtained from Amersham Biosciences (Arlington Heights, IL); \[^{3}H\]naltrindole (NTI; 33 Ci/mmol) and \[^{3}H\]DAMGO (54.5 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA); and \[^{3}H\]U-69,593 (65 Ci/mmol) was obtained from both commercial sources. All radioligands were diluted to the desired concentration in 5 mM HCl and stored at \(-20^\circ\text{C}\) until use. Naloxone, tetrodotoxin, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

**Tissue Isolation.** Intestinal segments were obtained from 30 weaned, outbred Yorkshire pigs of each sex (6–10 weeks of age; 10–18 kg body weight) that were not fasted before sacrifice. Animals

![Fig. 1. A and C, specific binding of 1 nM \[^{3}H\]saxitoxin in membrane fractions isolated from ileal submucosal (A) and cecal myenteric (C) plexuses. Nonselective binding was determined in the presence of 1 µM tetrodotoxin. B and D, specific \[^{3}H\]diprenorphine binding in membrane fractions that were enriched in \[^{3}H\]saxitoxin-binding sites from ileal submucosal (B) and cecal myenteric (D) plexuses. Bars represent the specific binding mean ± S.E.M. obtained in experiments using 2 to 10 replicates, using membranes isolated from two to five pigs (saxitoxin binding), or 9 to 11 replicates, using membranes isolated from four to six pigs (diprenorphine binding). PNS, postnuclear supernatant; S, supernatant; P, pellet; and Mic, microsomal fraction.](image-url)
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. Intestinal segments were collected as follows: jejunum, from the ligament of Trietz caudad for approximately 1.5 m; ileum/distal jejunum, from the ileocecal junction oral approximately 1.5 m; cecum, entire cecum; proximal colon, approximately 50 cm of the spiral (ascending) colon; and distal colon, from the rectum to approximately 50 cm oral. All segments were removed rapidly and placed in an ice-cold physiological salt solution (composition: 153 mM NaCl, 3 mM KCl, 1.2 mM CaCl$_2$, 1.5 mM CaHPO$_4$, 1.3 mM MgSO$_4$, and 11 mM d-glucose, pH 7.4). Subsequent tissue dissections were performed at 4°C. The mesenteric attachments were removed from all segments, which then were opened along the antimesenteric surface. The longitudinal and circular muscle layers were carefully separated from the underlying submucosa. These muscle layers were then diced into 5 × 5-mm cubes and stored at −70°C until neuronal membranes were isolated.

After removal of smooth muscle layers, each intestinal segment was rotated to expose the mucosa. The antimesenteric lymphoid tissue was excised from the segments of distal jejunum/ileum; Peyer’s patches in the proximal jejunum were not excised, because they comprised only a small percentage of the overall submucosal tissue. The intestinal mucosa was removed by gently scraping the mucosal surface with a razor blade. The submucosa prepared in this manner was inspected by secondary immunofluorescence for the presence of both submucosal plexuses with a primary antibody directed toward the neuronal marker PGP 9.5. The submucoea contained the internal submucosal plexus; the outer submucosal plexus often adhered to the circular muscle layer and thus, was partially included in both the myenteric and submucosal preparations.

**Isolation of Neuronally Enriched Membranes.** The isolation of neuronally enriched fractions was performed as described previously (Hildebrand et al., 1993). Submucosal membranes were isolated the same day on which they were harvested. Submucosal scrapings were diluted 1:10 in 50 mM Tris HCl (pH was adjusted to 7.4 with NaOH) and homogenized using a Polytron (Brinkmann Instruments, Westburg, NY; 25,000 rpm; three 8-s bursts). The homogenate was centrifuged at 800 g for 10 min, and the resulting pellet (P1) resulting from this procedure was also discarded; the supernatant (S1) was centrifuged at 48,000 g for 10 min. The final microsomal-synaptosomal pellet (P2) was re-suspended in 50 mM Tris and stored at −70°C until use in radioligand binding assays. In some cases, additional pellet (P3), supernatant (S2 and S3), and microsomal (Mic1 and Mic2) fractions were examined in [3H]STX- or [3H]diprenorphine binding assays.

Myenteric neuronal membranes were isolated no more than 72 h after tissue collection using a similar technique (Kostka et al., 1987). Minced tissue was diluted in 50 mM Tris HCl and homogenized as described above. The homogenate was then centrifuged at 800g for 10 min. The resulting PNS was centrifuged at 2500g for 10 min to yield a P1 fraction, and a second supernatant (S1) was subjected to centrifugation at 10,000g for 10 min to obtain the P2 fraction. As in the case of the submucosal plexus fractions, additional pellet, supernatant, and microsomal fractions were prepared to examine their radioligand binding characteristics. Neuronal enrichment of myenteric and submucosal P2 fractions was confirmed by [3H]STX binding at a radioligand concentration of 1 nM; nonspecific binding was determined by measuring [3H]STX binding in the presence of 1 μM tetrodotoxin. Protein concentrations were determined using the BCA protein assay (Pierce Chemical, Rockford, IL).

**Radioligand Binding Assays.** Isolated membranes were thawed on the day of the experiment and diluted, with 50 mM Tris, to a concentration of approximately 500 μg/mL. The actual protein concentration was determined from an aliquot of the diluted membrane used in the assay. Saturation analyses of OPR binding were performed using six concentrations of labeled ligand within the ranges indicated below. Nonspecific binding of OPR radioligands was determined by the binding of the labeled ligand in the presence of 1 μM unlabeled naloxone. The nonselective OPR antagonist diprenorphine possesses nearly equal affinities for all three OPR types, and therefore, [3H]diprenorphine (0.03–3 nM) was used to determine the density of total OPRs. Other ligands used in these studies included [3H]NTI (0.003–0.3 nM), a selective δ-OPR antagonist; [3H]DAMGO (0.03–10 nM), a highly selective μ-OPR agonist; and [3H]U-69,593 (0.1–10 nM), a selective κ-OPR agonist. All radioligand binding experiments were performed in a final volume of 500 μL with approximately 250 μg of protein. Binding assays were conducted at room temperature for 60 min to insure that equilibrium was achieved. Incubations were terminated by rapid vacuum filtration through GF/B glass fiber filters (Brandel Inc., Gaithersburg, MD) using a 24-well Brandel cell harvester. Filters were washed twice with 4 mL of 50 mM Tris (pH = 7.4). Filters were then submerged in Ecolite scintillation fluid (ICN Pharmaceuticals, Costa Mesa, CA). After a 12-h incubation period, the radioactivity of the samples was assessed by liquid scintillation spectroscopy.

**Data Analysis.** Specific opioid binding was calculated as the difference between binding in the presence (nonspecific) and absence (total) of 1 μM naloxone. The percentage of specific radioligand binding relative to total binding was also calculated. The integrity of each membrane preparation was determined by the density of both [3H]STX- and [3H]diprenorphine-specific binding sites. Tissue preparations (approximately 23% of the total examined) in which <50% of total [3H]STX or [3H]diprenorphine binding was, respectively displaced by tetrodotoxin or naloxone were omitted from the data analysis. Our results suggest that this screening procedure is effective at removing persistently low outliers, with the assumption that the receptor proteins in these discarded tissues were extensively degraded during the isolation procedure. Individual data points deviating more than two standard deviations from the mean (<5% of points) were also excluded from the analysis. Nonlinear regressions were used to determine the means and 95% confidence intervals (CI) for $B_{max}$ and $K_D$ of each radioligand in saturation analyses using the Prism 2.0 software program (GraphPad, San Diego, CA).

**TABLE 1** Specific [3H]diprenorphine binding

<table>
<thead>
<tr>
<th>Intestinal Region</th>
<th>Myenteric Plexus</th>
<th>Submucosal Plexus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_{max}$ (fmol/mg)</td>
<td>$K_D$ (nM)</td>
</tr>
<tr>
<td>Jejunum</td>
<td>73.63 (69.35–77.90)*</td>
<td>0.2973 (0.2397–0.3550)</td>
</tr>
<tr>
<td>Ileum</td>
<td>54.26 (39.34–69.18)</td>
<td>0.2178 (−0.009832–0.4454)</td>
</tr>
<tr>
<td>Cecum</td>
<td>77.14 (62.93–91.35)</td>
<td>0.2639 (0.08763–0.4303)</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>147.8 (107.1–189.1)</td>
<td>0.2901 (0.0227–0.5277)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>85.90 (77.90–94.40)</td>
<td>0.2949 (0.1981–0.3918)</td>
</tr>
</tbody>
</table>

* Values represent mean (95% CI) of 4 to 16 replications using membranes obtained from three to nine pigs.
Fig. 2. Representative data from saturation analyses of high-affinity \(^{3}H\)diprenorphine binding in submucosal and myenteric neuronal membranes isolated from various regions of the porcine intestinal tract. Specific (SB; open circles), nonspecific (NSB; filled triangles), and total (TB; filled squares) binding are shown. Abscissa indicates binding site density in fmol/mg protein, and ordinate indicates radioligand concentration. The thick line represents the result of the nonlinear regression fit of the specific binding data; results of these regressions are found in Table 1. Hatched and stippled lines represent the best fit of NSB and TB data to linear and nonlinear functions, respectively. Each point represents the mean ± S.E.; all calculations were performed on raw data, and then averaged. Data are representative of 4 to 16 replications using membranes obtained from three to nine pigs.
**Results**

Distribution of Specific $[^3H]$Saxitoxin (STX) - and $[^3H]$Diprenorphine-Binding Sites in Isolated Membrane Fractions. To determine the amounts of neuronal membranes in each fraction obtained from each intestinal subregion, the relative density of specific $[^3H]$STX-binding sites was determined. Figure 1 shows a representative fractional distribution from the submucosal plexus of the ileum (Fig. 1A) and the myenteric plexus of the cecum (Fig. 1C). $[^3H]$STX-binding sites were enriched in either P1, P2, or P3 fractions of cells isolated from either plexus along the length of the intestinal tract. Microsomal fractions and supernatants exhibited relatively less specific $[^3H]$STX binding. In these regions, the P2 fraction consistently expressed the highest density of $[^3H]$diprenorphine-binding sites in comparison to the other two pellet fractions (Fig. 1, B and D). This fraction was used in all subsequent determinations of radioligand binding.

$[^3H]$Diprenorphine-Binding Sites. $[^3H]$Diprenorphine bound with high affinity to specific sites in enteric neuronal membranes (P2 fractions) obtained from all intestinal segments (Table 1). In all regions, 1 μM naloxone displaced an average of 50 to 60% of total $[^3H]$diprenorphine binding (Fig. 2). Membranes from the submucosal and myenteric plexuses of the proximal colon had significantly more $[^3H]$diprenorphine-binding sites than the analogous subregions of jejunum, cecum, and distal colon (Table 1). Specific $[^3H]$diprenorphine-binding sites were not detected in mucosal homogenates obtained from the small intestines of two pigs (data not shown).

$[^3H]$NTI-Binding Sites. $[^3H]$NTI bound with high affinity to specific sites in myenteric and submucosal neuronal membrane homogenates obtained from all intestinal segments. With the exception of the distal colon myenteric plexus fraction, which displayed a slightly lower affinity for this radioligand than other regions, there were no significant variations in $[^3H]$NTI affinity for these sites along the length of the intestine (Table 2). Membranes from the myenteric plexuses of the proximal or distal colon had higher amounts of specific $[^3H]$NTI-binding sites than those from the myenteric plexuses of jejunum, ileum, or cecum. On the other hand, submucosal plexus fractions from the cecum and distal colon expressed lower amounts of $[^3H]$NTI-binding sites than those from the small intestine (Fig. 3).

$[^3H]$U-69,593-Binding Sites. $[^3H]$U-69,593 bound to specific sites in myenteric membranes from each intestinal segment with affinities in the nanomolar range. A detailed examination of the binding of this radioligand was limited by a relatively low signal-to-noise ratio; on average in all regions examined, <35% and <10% of total $[^3H]$U-69,593 binding was displaced by 1 μM naloxone in myenteric and submucosal neuronal membranes, respectively (Fig. 4). Specific $[^3H]$U-69,593-binding sites were at highest densities in myenteric neuronal membranes from the proximal colon and cecum (Table 3). None of the submucosal regions examined contained significant densities of U-69,593-binding sites.

$[^3H]$DAMGO-Binding Sites. Specific $[^3H]$DAMGO-binding sites were observed at relatively low densities in both myenteric and submucosal plexuses of the small intestine and cecum (Table 4). In these regions, specific $[^3H]$DAMGO binding accounted for <17% of total naloxone-displaceable binding (Fig. 5). In comparison, the radioligand bound with high affinity to specific sites in homogenates of porcine cerebral cortex under identical binding conditions ($K_D = 0.649$ with 95% CI of 0.282–1.02, and $B_{max} = 49.52$ with 95% CI of 41.73–57.32; $n = 6$ from three pigs).

**Discussion**

The present results demonstrate the expression of specific and saturable high-affinity binding sites for $[^3H]$diprenorphine and other radiolabeled opioids in membrane fractions from the ganglionated plexuses of the porcine small intestine and colon. Although these fractions were enriched in specific saxitoxin-binding sites and thus, are presumed to be comprised mainly of neuronal membranes, we cannot rule out the possible contributions of contaminating enteric glia and immune cells to the estimates of opioid-binding site densities obtained in these membrane preparations. The $K_D$ values determined for $[^3H]$diprenorphine and the highly selective OPR ligands, $[^3H]$NTI, $[^3H]$U-69,593, and $[^3H]$DAMGO, are within the range of their affinity constants for recombinantly expressed OPR types from rodents or humans (Raynor et al., 1994). Moreover, the affinity constants for $[^3H]$DAMGO in membranes derived from porcine enteric plexuses and porcine cerebral cortex were not significantly different. Considerable variability in the affinity estimates for some OPR radioligands was observed in membranes from some intestinal sites that possessed low densities of specific opioid binding. All binding studies were performed in Na⁺-free media, which favors the presence of high-affinity binding sites for both agonists and antagonists. Although the experimental conditions were selected to minimize the differences in the OPR binding of agonists and antagonists, it is possible that binding sites for the OPR agonists used in this study, i.e., U-69,593 or DAMGO, were at relatively low densities due to loss through preferential degradation or other processes. Of the radioligands used, only the enkephalin-based ligand $[^3H]$DAMGO displayed the lowest amounts of specific bind-

**Table 2**

Specific $[^3H]$naltrindole binding

<table>
<thead>
<tr>
<th>Intestinal Region</th>
<th>Myenteric Plexus</th>
<th>Submucosal Plexus</th>
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<tbody>
<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt; (fmol/mg)</td>
<td>K&lt;sub&gt;D&lt;/sub&gt; (nM)</td>
<td>B&lt;sub&gt;max&lt;/sub&gt; (fmol/mg)</td>
</tr>
<tr>
<td>Jejunum</td>
<td>29.02 (21.60–36.43)*</td>
<td>0.06196 (0.01867–0.1052)</td>
</tr>
<tr>
<td>Ileum</td>
<td>28.63 (26.89–30.36)</td>
<td>0.05549 (0.04617–0.06482)</td>
</tr>
<tr>
<td>Cecum</td>
<td>30.06 (25.81–34.31)</td>
<td>0.03211 (0.03112–0.07311)</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>55.38 (40.65–70.11)</td>
<td>0.1225 (0.05286–0.1921)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>60.71 (57.47–63.94)</td>
<td>0.1260 (0.1112–0.1407)</td>
</tr>
</tbody>
</table>

* Values represent mean (95% CI) of 4 to 19 replications using membranes obtained from three to eleven pigs.

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Fig. 3. Representative data from saturation analyses of high-affinity [3H]naltrindole binding in submucosal and myenteric neuronal membranes isolated from various regions of the porcine intestinal tract. Specific (SB, open circles), nonspecific (NSB; filled triangles), and total (TB; filled squares) binding are shown. Abscissa indicates binding site density in femtomoles per milligram of protein and ordinate indicates radioligand concentration. The thick line represents the result of the nonlinear regression fit of the specific binding data, results of these regressions are found in Table 2. Hatched and stippled lines represent the best fit of NSB and TB data to linear and nonlinear functions, respectively. Each point represents the mean ± S.E.; all calculations were performed on raw data and then averaged. Data are representative of 4 to 19 replications using membranes obtained from three to eleven pigs.
Fig. 4. Representative data from saturation analyses of high-affinity [3H]U-69,593 binding in submucosal and myenteric neuronal membranes isolated from various regions of the porcine intestinal tract. Specific (SB; open circles), nonspecific (NSB; filled triangles), and total (TB; filled squares) binding are shown. Abscissa indicates binding site density in femtomoles per milligram of protein, and ordinate indicates radioligand concentration. The thick line represents the result of the nonlinear regression fit of the specific binding data; results of these regressions are found in Table 3. Hatched and stippled lines represent the best fit of NSB and TB data to linear and nonlinear functions, respectively. Each point represents the mean ± S. E.; all calculations were performed on raw data and then averaged. Data are representative of 3 to 11 replications using membranes obtained from three to five pigs.
The presence of densities and with a more circumscribed distribution. The action appears to be mediated by a novel sheets mounted in Ussing chambers. In ileum, this action was evoked by transmural stimulation of mucosa-submucosa containing evidence supporting the presence of cells. There is no pharmacological or immunohistochemical profile specific to porcine myenteric neurons but not smooth muscle both of these OPR types are present (and occasionally colocalized) on porcine myenteric neurons but not smooth muscle. The present results suggest that δ-OPRs predominate along the length of the porcine intestinal tract, whereas κ- and μ-OPR binding sites are expressed at much lower densities and with a more circumscribed distribution. The presence of δ-OPRs has been determined in myenteric plexus preparations from several species, including the rat, dog, cat, baboon, and human (De Luca and Coupar, 1996). The relative expression of OPR-binding sites in the myenteric plexus of the porcine ileum is in agreement with the results of previous functional studies conducted in our laboratory. We have shown that δ- and κ-OPRs mediate opioid-induced decreases in neurogenic contractions evoked by field stimulation of a circular muscle-myenteric plexus preparation from the porcine small intestine and that immunoreactivities for both of these OPR types are present (and occasionally colocalized) on porcine myenteric neurons but not smooth muscle cells. There is no pharmacological or immunohistochemical evidence supporting the presence of μ-OPRs in this preparation (Poonyachoti et al., 2001a).

δ-OPRs appear to be associated with submucosal neurons of the small intestine or cecum of guinea pigs, mice, and dogs (De Luca and Coupar, 1996). In the porcine jejunum (Quito and Brown, 1991) and ileum (Poonyachoti et al., 2001b), peptidic δ- and μ-OPR agonists inhibit neurogenic ion transport evoked by transmural stimulation of mucosa-submucosa sheets mounted in Ussing chambers. In ileum, this action appears to be mediated by a novel δ-like OPR that is blocked preferentially by the δ-OPR antagonist 7-benzylidenenaltrexone. In addition, immunoreactivity for δ-OPRs, but not μ- or κ-OPRs, has been detected on neural elements in the inner submucosal plexus of porcine ileum (Poonyachoti et al., 2001b). These observations are consistent with the present finding of that δ-opioid-binding sites are the predominant OPR type present in ileal submucosal fractions. A few studies have provided evidence for the expression of opioid-binding sites on enterocytes (Lang et al., 1996; Nando et al., 2000). However, the lack of specific [3H]diprenorphine-binding sites in homogenates or membrane fractions of the ileal mucosa that do not exhibit [3H]HISTX binding suggests that OPRs are found only on neuronal membranes in the porcine intestine. This is in agreement with a previous radioligand binding study of OPRs in rat enterocytes (Gaginella et al., 1983). The predominance of δ-OPRs in the intestine of humans and many common laboratory animals suggests that this OPR type may mediate many of the effects of opioids in the small intestine and colon. This may be especially true for mucosal transport function, for which modulation by δ-OPRs appears to be conserved across species.

In neural fractions from many of the intestinal regions examined, the sum of the $B_{\text{max}}$ values obtained for the three OPR type-selective radioligands approximates the total population of opioid-binding sites as assessed through saturation binding analyses with [3H]diprenorphine, a nonselective, lipophilic opioid ligand. Myenteric plexus fractions from jejunum and proximal colon or submucosal plexus fractions from cecum and distal colon manifest specific [3H]diprenorphine-binding sites at densities that are greater than the sum total of the densities of [3H]NTI, [3H]U-69,593, and [3H]DAMGO-binding sites. This does not appear to be due to loss or removal of radioligands or receptor degradation for reasons addressed above. It is conceivable that additional naloxone-displaceable [3H]diprenorphine-binding sites exist in these plexuses that were not bound by the OPR type-selective radioligands used in this study. These may represent the

### TABLE 3
Specific [3H]U-69,593 binding

<table>
<thead>
<tr>
<th>Intestinal Region</th>
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<th>Submucosal Plexus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_{\text{max}}$</td>
<td>$K_D$</td>
</tr>
<tr>
<td>Jejunum</td>
<td>18.80 (15.73–21.87)</td>
<td>1.597 (0.8535–2.341)</td>
</tr>
<tr>
<td>Ileum</td>
<td>15.52 (11.18–19.87)</td>
<td>1.266 (0.2218–2.311)</td>
</tr>
<tr>
<td>Cecum</td>
<td>41.75 (35.45–48.05)</td>
<td>1.341 (0.7562–1.926)</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>46.81 (33.48–60.15)</td>
<td>2.727 (0.7662–4.687)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>7.696 (6.175–9.216)</td>
<td>0.3368 (0.07919–0.5944)</td>
</tr>
</tbody>
</table>

N.A., not applicable; N.D., not determined.

* Values represent mean (95% CI) of 3 to 11 replications using membranes obtained from three to five pigs.

### TABLE 4
Specific [3H]DAMGO Binding

<table>
<thead>
<tr>
<th>Intestinal Region</th>
<th>Myenteric Plexus</th>
<th>Submucosal Plexus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_{\text{max}}$</td>
<td>$K_D$</td>
</tr>
<tr>
<td>Ileum</td>
<td>3.181 (0.8803–5.482)</td>
<td>4.131 (–2.883–11.14)</td>
</tr>
<tr>
<td>Cecum</td>
<td>6.151 (3.807–8.575)</td>
<td>1.715 (–0.4195–3.850)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>N.A.</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

N.A., not applicable; N.D., not determined.

* Values represent mean (95% CI) of 3 to 11 replications using membranes obtained from three to six pigs.
Fig. 5. Representative data from saturation analyses of high-affinity [3H]DAMGO binding in submucosal and myenteric neuronal membranes isolated from various regions of the porcine intestinal tract. Specific (SB; open circles), nonspecific (NSB; filled triangles), and total (TB; filled squares) binding are shown. Abscissa indicates binding site density in fmol/mg protein, and ordinate indicates radioligand concentration. The thick line represents the result of the nonlinear regression fit of the specific binding data; results of these regressions are found in Table 4. Other lines represent the best fit of NSB and TB data to linear and nonlinear functions, respectively. Each point represents the mean ± S.E.; all calculations were performed on raw data and then averaged. Data are representative of 3 to 11 replications using membranes obtained from three to six pigs.
putative “U-69,593-insensitive” ε- or η2-OPR, for example. This receptor binds [3H]diprenorphine with high affinity, but has low affinity for [3H]U-69,593 (Nock et al., 1990). Because [3H]U-69,593 was used over a narrow concentration range chosen to detect high-affinity κ-OPR sites, these latter receptors might be labeled only by [3H]diprenorphine. Putative κε/ε-binding sites have been detected in guinea pig ileum (Webster et al., 1993).

Enkephalins have been proposed to be the endogenous ligands for δ-OPRs and they are expressed at high concentrations in neurons of the porcine myenteric plexus (Porcher et al., 2000). In this location, the expression of δ-OPRs and their cognate ligands is consistent with a physiological role for enkephalins in the neuromodulation of intestinal motor function. Although δ-OPRs also appear to be present in the submucosal plexuses of the porcine intestine, enkephalin-like immunoreactivity is sparse or undetectable in these locations (Porcher et al., 2000). An as yet unidentified endogenous ligand may be expressed in the submucosa that interacts with δ-OPRs associated with submucosal neurons; this could include members of the endomorphin peptide family that have been shown to activate the porcine submucosal OPR and decrease neurogenic intestinal transport (Poonyachoti et al., 2001b). In addition to their importance in investigations of opioid receptor biology, enteric OPRs present in the myenteric and submucosal plexuses may represent therapeutic targets for the treatment of intestinal motility disorders, alleviation of diarrheal disease, modulation of intestinal host defense processes, and relief of abdominal pain.

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References

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