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Pharmacological characterization of a 7-benzylidenenaltrexone-preferring opioid receptor in porcine ileal submucosa

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1 In the intestine, opioids produce antidiarrhoeal and constipating actions that are mediated by enteric neurones. Through interactions with opioid receptors (ORs) on submucosal neurones, opioids suppress active ion transport evoked by transmural electrical stimulation (TES) in mucosa–submucosa sheets from the porcine ileum. In this study, we examined the pharmacological characteristics of the previously described OR, which is sensitive to the δ_1-OR antagonist 7-benzylidenenaltrexone and modulates neurogenic transepithelial ion transport in this tissue preparation.

2 Increases in short-circuit current (I_{sc}, a measure of active anion transport) evoked by TES in ileal mucosa–submucosa sheets were inhibited by opioid agonists possessing high selectivity for either δ- or μ-ORs including [D-Pen^{2,5}]enkephalin (DPDPE), [D-Ala^2, Glu^4]deltorphin II, and [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin (DAMGO).

3 As determined by the Schild analysis, the actions of these agonists were competitively inhibited by 7-benzylidenenaltrexone. The nonequilibrium μ-OR antagonist β-funaltrexamine inhibited the actions of DAMGO only at a high concentration (1 μM) but did not alter DPDPE or deltorphin II action. At concentrations up to 10 μM, the nonequilibrium δ-OR antagonist naltrindole 5′-isothiocyanate did not alter the actions of δ- or μ-OR agonists.

4 Radioligand binding analyses of neuronal homogenates from the ileal submucosa revealed that the nonselective OR ligand [^{3}H]diprenorphine bound to two populations of specific binding sites. One of these sites possessed binding characteristics similar to the δ-OR.

5 In summary, neurogenic ion transport in the porcine intestine is modulated by an OR which shares pharmacological characteristics of both μ- and δ-ORs and may represent a novel receptor entity.
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Keywords: Enteric nervous system; δ-opioid receptor; intestinal secretion; neurogenic ion transport; antidiarrhoeal action; β-funaltrexamine; 7-benzylidenenaltrexone; naltrindole 5′-isothiocyanate; diprenorphine; [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin

Abbreviations: BNTX, 7-benzylidenenaltrexone; DAMGO, [D-Ala^2, *N*-methyl-Phe^4, Gly^5-ol]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; DPN, diprenorphine; β-FNA, β-funaltrexamine; G_t, tissue electrical conductance; I_{sc}, short-circuit current; 5′-NTII, naltrindole 5′-isothiocyanate; OR, opioid receptor; TAN-67, 2-methyl-4*aa*-(3-hydroxyphenyl)-1,2,3,4,4*a*,5,12,12*a*α-octahydroquinolino[2,3,3-]isoquinoline; TES, transmural electrical stimulation

Introduction

For millenia, opium has been employed to alleviate diarrhoeal disorders and opioids continue to be the most effective class of antidiarrhoeal drugs known (Schiller, 1995). Moreover, drugs capable of decreasing the degradation of endogenous opioid peptides in the intestine, such as the ‘enkephalinase’ inhibitor acetorphan, have been shown to be effective in the treatment of paediatric diarrhoea (Salazar-Lindo *et al*., 2000). The profound constipating actions of opioid analgesics complicate the use of these drugs in palliating chronic painful conditions (Pappagallo, 2001). The antidiarrhoeal and constipating actions of opioids are due in part to the inhibition of both intestinal propulsion and active transepithelial ion transport meditated by opioid receptors (ORs) expressed by enteric neurones (De Luca & Coupar, 1996). Agonist stimulation of these receptors is associated with reduced enteric neuronal transmission through increased neuronal K^+ conductance (Mihara & North, 1986) or decreased Ca^{2+} conductance (Surprenant *et al*., 1990). In several species, including the mouse (Sheldon *et al*., 1990), guinea-pig (Surprenant *et al*., 1990), and pig (Poonyachoti *et al*., 2001), the intestinal antisecretory actions of opioids appear to be mediated by δ-ORs expressed on submucosal neurones. In the porcine ileum, submucosal δ-ORs mediate opioid-induced suppression of neurogenic secretion produced by histamine (Poonyachoti & Brown, 2001), 5-hydroxytryptamine (Green & Brown, 2002), kinins (Green *et al*., 2003), tryptase (Green *et al*., 2000), and intestinal anaphylaxis (Poonyachoti & Brown, 2001). Submucosal neurones in the porcine ileum that display δ-OR-like immunoreactivity have been found to coexpress immunoreactivities for vanilloid VR_1 receptors and calcitonin gene-related peptide as well; these latter neuronal markers are commonly associated with primary afferent neurones (Poonyachoti *et al*., 2002).

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δ-Opioid receptors have been characterized pharmacologically using both *in vitro* and *in vivo* models. Pharmacological studies *in vivo* have uncovered two putative δ-OR subtypes in the central nervous system, as well as evidence for an interaction between δ- and μ-ORs. Receptor subtypes were classified using both selective antagonism and by the absence of agonist cross-tolerance. These studies defined δ_1-ORs as those selective for the δ-OR antagonist 7-benzylidenenaltrexone (BNTX) and the δ-OR agonists [D-Pen^2, D-Pen^5]-enkephalin (DPDPE) and TAN-67, and δ_2-ORs as those preferring the δ-OR agonist deltorphin II and δ-OR antagonist naltriben. In addition to these specific agonists and competitive antagonists, selective nonequilibrium antagonists for putative δ_1- and δ_2-OR subtypes have been developed including [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE) and naltrindole-5′-isothiocyanate (5′-NTII), respectively (Zaki *et al.*, 1996). However, δ-OR subtypes have not been clearly identified by extensive analyses *in vitro* of the single-cloned δ-OR or of native δ-ORs in transformed cells. The recombinant δ-OR exhibits a high affinity for both δ_1- and δ_2-OR ligands (Clark *et al.*, 1997). In mice with disruption of the δ-OR gene, the spinal analgesic actions of δ-opioid agonists are lost, although both deltorphin II and DPDPE retain analgesic activity at the supraspinal level (Zhu *et al.*, 1999). These data suggest that there are additional factors present *in vivo* that may be required for expression of δ-OR subtypes. Complicating this classification scheme is the potentiation of μ-OR action by δ-OR agonists observed *in vivo* (Traynor & Elliott, 1993), the presence of δ-OR in functional heterodimers with other OR types (Jordan & Devi, 1999; George *et al.*, 2000), and the possibility of splice variants that may alter the pharmacology of the receptor (Rossi *et al.*, 1997).

We have reported previously that δ-like ORs are present in the isolated mucosa–submucosa of porcine ileum that mediate opioid-induced inhibition of neurogenic intestinal ion transport (Quito & Brown, 1991; Poonyachoti *et al.*, 2001). Despite the apparent absence of neuronal μ-OR immunoreactivity (Poonyachoti *et al.*, 2001) or high-affinity μ-opioid binding sites (Townsend & Brown, 2002) in this preparation, the selective μ-OR agonists DAMGO, PL017, and endomorphin II manifest antisecretory potencies and effectiveness similar to that of DPDPE and other peptidic δ-OR agonists. However, the antisecretory potencies of morphine or the synthetic δ-OR agonist SNC-80 are >100-fold lower than those of the peptidic OR agonists. Moreover, this receptor displays a preferential affinity for BNTX in comparison with other selective δ-OR antagonists. These results suggest the possibility that an atypical OR is expressed in the intestinal submucosa. In the present study, we extended the initial characterization of this submucosal OR through the use of additional, selective OR agonists and antagonists as well as nonequilibrium OR antagonists in a functional assay of neurogenic ion transport and an analysis of specific opioid binding sites in submucosal neuronal homogenates.

Methods

Drugs and reagents

DPDPE, [D-Ala^2, *N*-methyl-Phe^4, Gly^5-ol]enkephalin (DAMGO), and [D-Ala^2, Glu^4]deltorphin II were obtained from Bachem (Torrance, CA, U.S.A.). 5′-NTII, β-funaltrexamine (β-FNA), naltriben, BNTX, and naltrindole were generously provided by Dr Philip S. Portoghese (Department of Medicinal Chemistry, University of Minnesota College of Pharmacy). Naloxone, (+)-4-[(αR)-α((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl-3-methoxybenzyl)-*N*,*N*-diethylbenzamide (SNC-80), and tetrodotoxin were purchased from Sigma-RBI (St Louis, MO, U.S.A.). 2-Methyl-4*aa*-(3-hydroxyphenyl)-1,2,3,4,4*a*,5,12,12*a*α-octahydroquinolino[2,3,3-]isoquinoline (TAN-67) was purchased from Tocris Cookson (Baldwin, MO, U.S.A.). Morphine sulphate was obtained from Mallinckrodt (Hazelwood, MO, U.S.A.). All drugs used in functional assays were serially diluted with distilled water. Stock solutions of drugs were made in water, with the exceptions of β-FNA, which was dissolved in methanol, and both SNC-80 and 5′-NTII, which were dissolved in dimethylsulphoxide. Neither organic solvent had significant effects on mucosal electrical responses to electrical transmural stimulation. [^{3}H]Diprenorphine ([^{3}H]DPN; 50 Ci mmol^{-1}) was purchased from Perkin-Elmer Life Sciences (Boston, MA, U.S.A.) and [^{3}H]saxitoxin ([^{3}H]STX; 14.9 Ci mmol^{-1}) was obtained from Amersham Biosciences (Arlington Heights, IL, U.S.A.).

Animals and tissue isolation

Intestinal tissues were obtained from weaned, outbred Yorkshire pigs of each sex that were 6–10 weeks of age and weighed between 10 and 18 kg. Pigs were housed for 3–7 days in a holding room with constant access to water and standard nonmedicated pig feed and were not fasted prior to killing.

Animals were sedated with an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol®; 8 mg kg^{-1}, Fort Dodge Laboratories, Fort Dodge, IA, U.S.A.), in combination with xylazine (8 mg kg^{-1}). They 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 extending orad from the ileocaecal junction was removed and incised along the antimesenteric border.

Measurement of ion transport

The serosa and smooth muscle layers of an excised ileal segment were removed by blunt dissection and the remaining submucosa–mucosa was mounted between two lucite Ussing-type half chambers; two strips of aluminium foil were placed diagonally on opposite sides of the tissue, these foil strips covered about half of the 2 cm^2 flux area. Mucosal sheets were bathed in a physiological salt solution (composition in mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 0.5; $NaHCO_3$, 25; NaH_2PO_4, 1.0) at pH 7.4 and gassed with 5% CO_2 in O_2 at 39°C. D-Glucose and mannitol were added to the contraluminal and luminal media at 10 mM, respectively. The short-circuit current (I_{sc}), a measure of net, electrogenic ion transport, was monitored by an automatic voltage clamp.

Throughout each experiment, the transepithelial voltage was periodically clamped to ± 5 mV, and the resulting change in I_{sc} was used to calculate the tissue conductance (G_t) by Ohm's

law. After an equilibration period of 60 min, transmural electrical stimulation (TES; 300 bipolar current pulses at 10 Hz, 0.5 ms pulse duration, $2.1\,mA\,cm^{-2}$) was delivered through the aluminium foil electrodes by a Model S-88 stimulator and Model SIU-5 stimulus isolation unit (Astro-Med Grass Instruments, Quincy, MA, U.S.A.). Each stimulus period was followed by the contraluminal addition of 40 μmol of glucose. After three successive TES-induced I_{sc} elevations produced peak I_{sc} changes of equal ($\pm$10%) magnitude, agonist concentration–effect relationships were determined.

Pharmacological characterization of opioid receptors

Cumulative concentration–effect curves were determined for opioid agonists added to the contraluminal bathing medium. Agonists were administered approximately 2 min after each 30 s interval of TES, and each stimulation period was separated by 10 min to allow for return of the I_{sc} to baseline values. Competitive antagonists were added to the contraluminal bathing medium prior to TES delivery and the effects of antagonists on the TES-induced I_{sc} elevation in the absence of agonist were assessed; after this initial TES interval, agonists were added in increasing concentrations to the contraluminal bathing medium. Nonequilibrium, alkylating antagonists were added to the contraluminal bathing medium for 30 min and then washed out prior to TES delivery and the assessment of agonist concentration–effect relationships. After determination of each concentration–effect relationship, the effects of two additional TES deliveries on I_{sc} were measured in order to detect any tissue tachyphylaxis to the stimulation paradigm; peak changes in I_{sc} occurring in response to two successive TES deliveries had to be of equal magnitude if data obtained from a given tissue were included in the final analysis. The viability of each mucosal sheet was also assessed at the end of each experiment through measurement of peak changes in I_{sc} evoked by the contraluminal addition of 10 μM carbachol and the luminal addition of 40 μmol of glucose.

Submucosal neuronal membrane preparation

Ileal segments were isolated and stripped of muscle layers as described above. The overlying mucosa was removed, leaving the submucosal connective tissue and associated neuronal plexuses. Each sheet of submucosa was diced into small pieces and diluted in either 50 mM Na^+-free Tris-HCl or Krebs–HEPES buffer (composition in mM: NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgCl_2$, 1.2 and HEPES, 25); both buffers were maintained at pH 7.4. These tissues were then homogenized using a Brinkman Polytron (three 8 s pulses at 25,000 r.p.m.); the resulting homogenate was subjected to several differential centrifugation steps, to yield a membrane preparation (P2 fraction) enriched in neuronal membranes as described previously (Townsend & Brown, 2002). Protein concentrations were determined with a BCA Protein assay kit (Pierce Chemical, Rockford, IL, U.S.A.).

Radioligand binding assays

Isolated neuronal membranes were thawed on the day of the experiment and diluted, in the Tris or Krebs–HEPES buffers described above, to a final concentration of $500\,\mu g\,ml^{-1}$. The actual protein concentration was determined from an aliquot of the diluted membrane used in each assay. Specific binding sites for the neuronal Na^+ channel blocker saxitoxin (STX) were detected with [^{3}H]STX at a concentration of 1 nM; nonspecific binding was determined by the measurement of [^{3}H]STX binding in the presence of 1 μM of unlabelled tetrodotoxin. Saturation analyses of specific opioid binding sites were performed using the nonselective OR antagonist [^{3}H]diprenorphine (DPN; 0.03–3 nM); nonspecific binding was determined in the presence of 1 μM unlabelled naloxone. Affinities (K_i) of opioid ligands were determined by their displacement of [^{3}H]DPN (1 nM) from specific binding sites. All binding assays were initiated by the addition of membranes to tubes containing radioligand and any unlabelled ligand present. Assays were allowed to incubate for 60 min at room temperature before rapid filtration of unbound ligands. Glass fibre filters were then washed twice with 4 ml of cold Tris or Krebs–HEPES buffer. Filters were then submerged in scintillation fluid and counted approximately 12 h later.

Data analysis

In each tissue preparation, opioid inhibition of TES-induced peak changes in I_{sc} is expressed as a percentage change from the average of the initial three I_{sc} responses to TES determined prior to agonist addition. Concentration–effect relationships were analysed by nonlinear regression. The resulting pIC_{50} value and maximal response from each tissue were pooled and averaged to provide the values reported. The same procedure was used to analyse the results of the receptor alkylation studies. Schild analysis was performed by averaging IC_{50} values at a given antagonist concentration obtained from 4 to 11 tissues; the resulting mean IC_{50} values obtained in antagonist-treated and untreated (control) tissues were used to calculate concentration ratios. These data were analysed by a linear regression; if the 95% confidence interval included unity but excluded zero, the slope was set equal to one and the analysis was repeated to determine the intercept (pA_2) that best fit the data.

In saturation analyses, specific binding was determined by calculating the difference between [^{3}H]DPN or [^{3}H]STX binding in the presence and absence of 1 μM naloxone or tetrodotoxin, respectively. The resulting data were averaged for each concentration then analysed by nonlinear regression. Similarly, the data from the [^{3}H]DPN displacement studies were averaged and analysed using nonlinear regression; for these studies K_i values were calculated by the method of Cheng & Prusoff (1973). In all cases, a single binding site model was chosen, unless a two-site model gave a significantly better fit by F-test ($P<0.05$). All nonlinear regressions were performed using the Prism statistical software package (version 3.0c; GraphPad, San Diego, CA, U.S.A.).

Comparisons between a control mean and a single treatment mean were made with a two-tailed, unpaired Student's *t*-test. Comparisons of multiple means were made by one-way analysis of variance followed by Tukey's test. In all cases, the limit for statistical significance was set at $P<0.05$. These statistical analyses were performed using JMP statistical software (SAS Institute, Cary, NC, U.S.A.).

Results

Effects of opioids on short-circuit current evoked by electrical transmural stimulation in ileal mucosa–submucosa sheets

The tissues ($n = 422$) used in this study manifested a baseline I_{sc} of $-3.2 \pm 0.8\,\mu A\,cm^{-2}$ and an electrical conductance (G_t) of $17.8 \pm 0.2\,mS\,cm^{-2}$. TES produced a transient peak increase in I_{sc} that averaged $43.3 \pm 1\,\mu A\,cm^{-2}$ relative to baseline values. Repeated stimulus delivery in the absence of agonist addition resulted in an $18.2 \pm 5.4\,\mu A\,cm^{-2}$ increase in baseline I_{sc} and a decrease in G_t of $1.0 \pm 0.4\,mS\,cm^{-2}$ ($n = 8$) over the time course of an experiment; these values were not significantly altered by any of the agonists used in these studies (ANOVA; Dunnett's post-test, $P < 0.05$). Peak increases in I_{sc} produced by TES were inhibited in a concentration-dependent manner by the δ-OR-selective agonists DPDPE and deltorphin II as well as the μ-OR-selective agonist DAMGO (Figure 1a; Table 1). Indeed, DAMGO appeared to be more effective than either deltorphin II, DPDPE, or TAN-67 (ANOVA; Tukey's post-test, $P < 0.05$). As previously reported (Poonyachoti *et al.*, 2001), the nonpeptidic δ-OR agonist SNC-80 also inhibited mucosal I_{sc} responses to TES, but was significantly less potent than any of the other opioid agonists examined. In contrast, the nonpeptidic agonist TAN-67 was a relatively potent inhibitor of TES-evoked I_{sc} elevations (Figure 1b; Table 1). At concentrations exceeding 1 μM, it produced a transient increase in I_{sc} relative to baseline values prior to TES delivery.

Table 1 Potency (expressed as pIC_{50} values) and maximal inhibitory action (as % decrease from control values) of opioid agonists on TES-evoked neurogenic ion transport in the porcine ileal mucosa

Agonist	*Receptor selectivity*	*pIC_{50} (mean ± s.e.m.)*	*Maximal inhibition (mean ± s.e.m.)*	n *tissues (from* N *pigs)*
Deltorphin II	δ	7.95±0.13	46.0±2.7	30 (28)
TAN-67	δ	7.88±0.39	40.7±4.9	7 (6)
DPDPE	δ	7.42±0.15	49.2±2.5	25 (21)
DAMGO	μ	6.98±0.08	60.6±1.8	38 (31)
SNC-80	δ	5.79±0.14	60.0±3.6	7 (6)

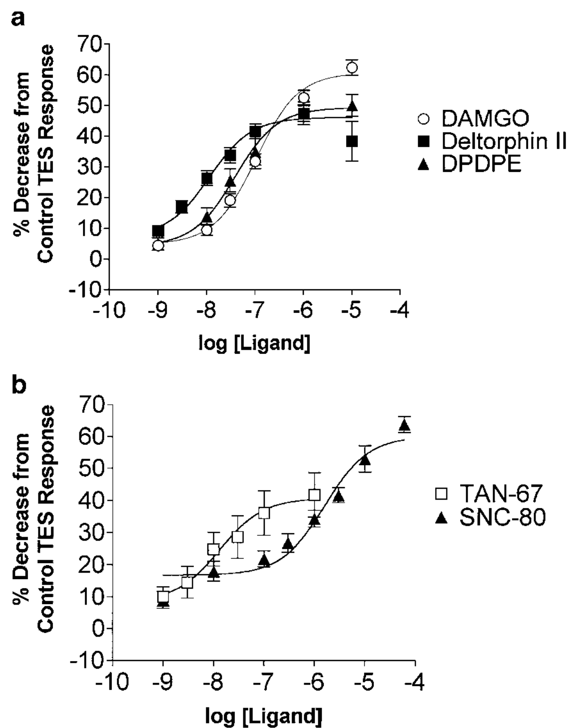

Figure 1 Concentration-dependent inhibition of TES-evoked increases in short-circuit current (I_{sc}) by OR agonists. (Top) The inhibitory actions of the peptidic opioid agonists deltorphin II (filled squares), DPDPE (filled triangles), and DAMGO (open circles) are shown. (Bottom) The inhibitory actions of the nonpeptidic δ-opioid agonists TAN-67 (open squares) and SNC-80 (filled triangles) are shown. Data are expressed as the percentage decrease in neurogenic I_{sc} response to TES relative to predrug control responses; the abscissa indicates $\log_{10}$ molar agonist concentration. Each point represents the mean ± s.e.m. of responses determined in 7–39 tissues from 6–30 pigs. The integrated results of the data analysis can be found in Table 1.

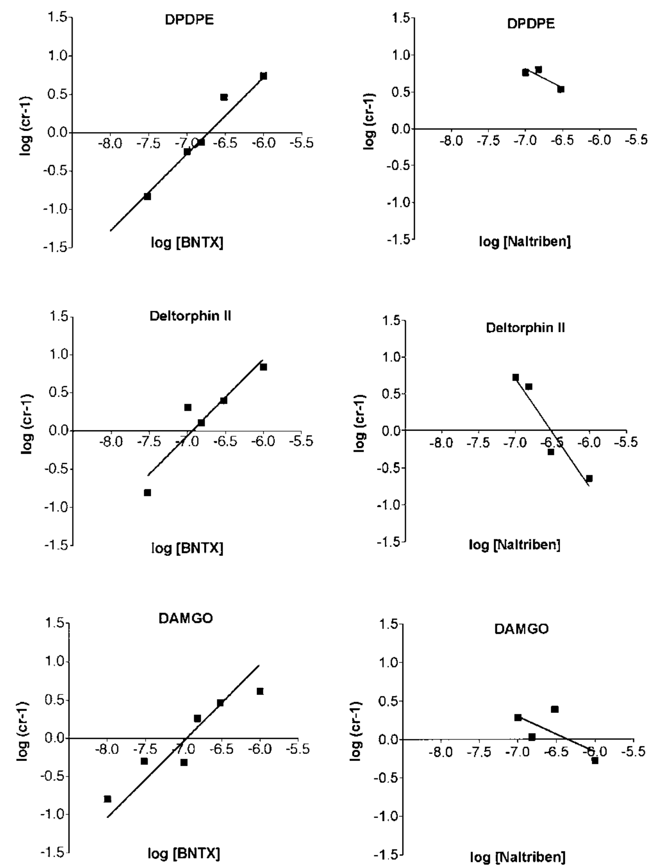

Figure 2 Schild analyses of the antagonistic actions of BNTX and naltriben on the antisecretory effects of selective opioid agonists. (Left) BNTX appeared to interact competitively with each opioid agonist, with pA_2 values against DPDPE (top; 6.96±0.11), deltorphin II (middle; 6.93±0.07), and DAMGO (bottom; 6.72±0.10) that did not differ significantly. (Right) Affinity estimates could not be made for naltriben, which did not appear to interact competitively with each agonist tested. Each point represents the average agonist IC_{50} value determined at fixed antagonist concentrations and the mean IC_{50} value for each agonist in control tissues untreated with antagonists as determined in 3–11 tissues from 3–11 pigs.

Effects of selective opioid antagonists

To examine the relative potencies of selective OR agonists in mucosa–submucosa sheets, Schild analyses were performed for the δ-OR antagonists BNTX, naltriben, and naltrindole in inhibiting the antisecretory actions of DPDPE, deltorphin II, and DAMGO. BNTX appeared to interact competitively with all three agonists, manifesting respective pA_2 values of 6.96±0.11, 6.72±0.07, and 6.93±0.10 *versus* DAMGO, DPDPE, and deltorphin II that did not differ significantly (Figure 2). On the other hand, naltriben failed to interact in a competitive manner with any of the three agonists (Figure 2), and naltrindole did not appear to antagonize the actions of DAMGO or deltorphin II in a competitive fashion (data not shown).

Effects of selective nonequilibrium opioid antagonists

The potencies and maximal inhibitory effects of deltorphin II or DPDPE were not significantly altered in tissues pretreated with either of the selective nonequilibrium opioid antagonists β-FNA or 5′-NTII at contraluminal concentrations ranging from 0.1 to 10 μM (Figure 3, Table 2). Similarly, the inhibitory action of DAMGO remained unaffected by 5′-NTII at concentrations up to 10 μM (Figure 4, Table 2) or by β-FNA or naloxonazine at a contraluminal concentration of 0.1 μM (data not shown). Concentrations of 1 and 10 μM β-FNA produced a significant rightward shift in the concentration–effect relationship for DAMGO that was not accompanied by a change in its maximum effect (Figure 4, Table 2).

Analysis of specific opioid binding in submucosal neuronal membranes

To further characterize the OR present in the ileal submucosa, we analysed the binding of the nonselective OR receptor antagonist [^{3}H]DPN to a submucosal neuronal membrane homogenate enriched in specific [^{3}H]STX binding sites. A single, high-affinity binding site for [^{3}H]DPN was detected in homogenates incubated in either Na^+-free Tris ($n=4$) or Na^+-replete Krebs–HEPES buffer ($n=8$); its affinity (0.2±0.07 *versus* 0.6±0.2 nM) and the density (78±7 *versus* 105±15 fmol mg^{-1} protein) of [^{3}H]DPN binding sites was not significantly different under either condition (Figure 5). BNTX displaced 1 nM [^{3}H]DPN from a single binding site with nanomolar affinity in either Tris or Krebs–HEPES buffers (Figure 6; Table 3). This binding site accounted for approximately 70% of total [^{3}H]DPN binding to these membranes; as this degree of displacement was similar to that observed in the presence of 1 μM naloxone, it represents total specific [^{3}H]DPN binding. Unlike BNTX, naltriben displaced approximately 50% of total specific [^{3}H]DPN binding from a high-affinity site and the remainder from a lower affinity site (Figure 6, Table 3).

The δ-OR agonists DPDPE and deltorphin II displaced [^{3}H]DPN from a single high-affinity binding site in neural membranes incubated in Na^+-free Tris buffer and predictably exhibited a 7- to 10-fold lower affinity for this site in membranes incubated in Na^+-replete Krebs–HEPES buffer (Figure 7; Table 3). However, they maximally displaced only 30–40% of total [^{3}H]DPN binding, even at relatively high concentrations. The nonpeptide δ-OR agonists SNC-80 and TAN-67 also maximally displaced 40–45% of total [^{3}H]DPN binding. In contrast to the peptide agonists however, a second binding site for these agonists became apparent in membranes incubated with Krebs–HEPES buffer (Figure 8, Table 3). This additional binding did not have a significant effect on the maximal level of [^{3}H]DPN displacement. Combinations of saturating concentrations of DPDPE, deltorphin II, and SNC-80 provided no additional displacement (data not shown). The μ-OR agonists DAMGO (Figure 7; Table 3) and morphine (Figure 8; Table 3) maximally displaced approximately 70% of total [^{3}H]DPN binding. Morphine displaced [^{3}H]DPN from a single site, whereas DAMGO appeared to bind to two sites; these sites were of 10-fold lower affinity in membranes incubated in Krebs–HEPES buffer compared to those incubated in Na^+-freeTris.

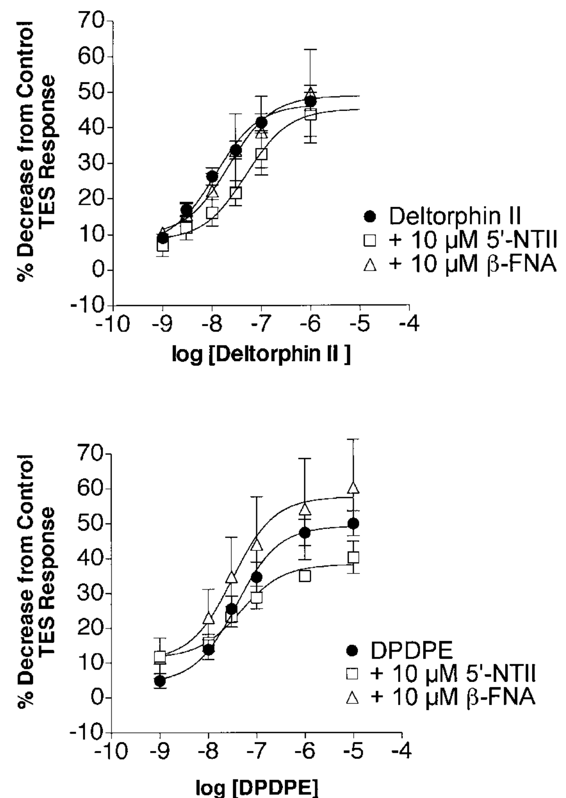

Figure 3 Effects of selective nonequilibrium opioid antagonists β-FNA and 5′-NTII on the antisecretory activity of selective δ-OR agonists. Tissues were preincubated with each antagonist at the concentrations indicated for 30 min. After washing tissues to remove unbound antagonist, the concentration-related effects of the selective δ-OR agonists deltorphin II (top) and DPDPE (bottom) in decreasing mucosal I_{sc} responses to electrical transmural stimulation were determined. Data are expressed as the percentage decrease in neurogenic I_{sc} response to TES relative to preagonist control responses; the abscissa indicates $\log_{10}$ molar agonist concentration. Agonist concentration–effect curves obtained from control tissues untreated with antagonists (filled circles) as seen in Figure 1 are included for comparison. Each point represents the mean±s.e.m. of peak I_{sc} responses in three tissues from three pigs. The integrated results of the data analysis can be found in Table 2.

Discussion

Previous pharmacological, immunohistochemical, and radioligand binding studies have demonstrated the presence of

Table 2 Effects of nonequilibrium opioid receptor antagonists on the potency (expressed as pIC_{50} values) and maximum inhibitory action (as % decrease from control values) of peptidic opioid agonists in the porcine ileal mucosa–submucosa

Agonist	β-*FNA*				5′-*NTII*			
	pIC₅₀±s.e.m.	*Maximal inhibition±s.e.m.*	*[β-FNA] (μM)*	n *tissues (from* N *pigs)*	*pIC₅₀±s.e.m.*	*Maximal inhibition±s.e.m.*	*[5′-NTII] (μM)*	n *tissues (from* N *pigs)*
Deltorphin II	7.65±0.40	48.8±7.1	10	3 (3)	7.30±0.27	45.1±5.4	10	3 (3)
DPDPE	7.50±0.43	57.5±7.6	10	3 (3)	7.33±0.20	38.2±2.1	10	3 (3)
DAMGO	5.64±0.48	48.2±7.7	1	9 (7)	7.18±0.66	53.4±9.0	10	3 (3)

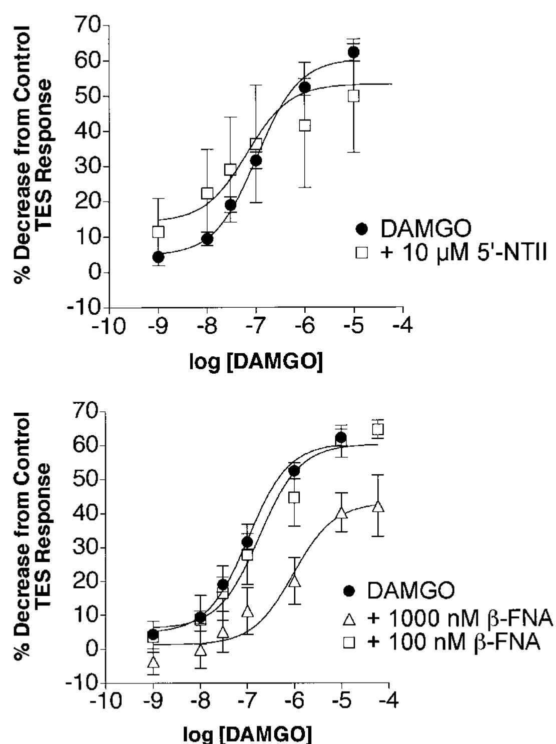

Figure 4 Effects of selective nonequilibrium opioid antagonists 5′-NTII (top) and *β*-FNA (bottom) on the antisecretory activity of DAMGO. Agonist concentration–effect curves obtained from control tissues untreated with antagonists (filled circles) as seen in Figure 1 are included for comparison. Data are expressed as the percentage decrease in neurogenic I_{sc} response to TES relative to pre-DAMGO control responses; the abscissa indicates $\log_{10}$ molar DAMGO concentration. Each point represents the mean±s.e.m. of peak I_{sc} responses in three to nine tissues from three to seven pigs. The integrated results of the data analysis can be found in Table 2.

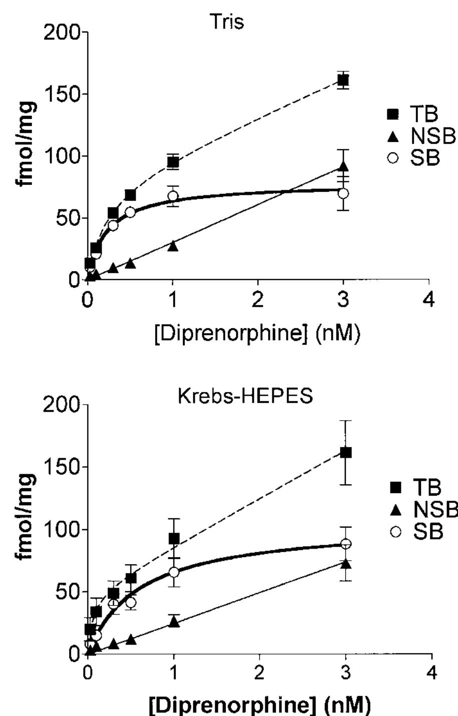

Figure 5 Saturation analysis of [³H]DPN binding in submucosal neuronal membranes incubated in either Na^+-free Tris buffer (top) or Na^+-containing Krebs–HEPES buffer (bottom). Total binding (TB; filled squares), nonspecific binding (NSB; filled triangles), and specific binding (SB; open circles) are shown. The ordinate indicates the binding site density in fmol mg^{-1} protein, and the abscissa indicates nanomolar concentration of the radioligand. Nonlinear regression analysis of specific binding sites in both solutions indicated the presence of a single high-affinity binding site having K_d = 0.23 (0.075–0.38) nM and 0.60 (0.14–1.10) nM in Tris (n = 4) and Krebs–HEPES (n = 8) solutions, respectively. Binding densities were not significantly different in membranes bathed in either solution (77.8±7.0 and 105±15 fmol mg^{-1} protein in Tris and Krebs–HEPES, respectively). Each point represents the mean± s.e.m. of [³H]DPN binding derived from independent binding experiments in four or eight assays, each assay using neuronal membranes obtained from separate pigs. Thin dashed and solid lines represent the best nonlinear and linear fit to the total and nonspecific binding, respectively.

δ-ORs and the relative absence of *μ*-ORs in the mucosa and submucosa of the porcine ileum (Poonyachoti *et al.*, 2001; Townsend & Brown, 2002). Nevertheless, several selective *μ*-OR agonists are quite potent and effective inhibitors of neurogenic ion transport in this tissue preparation. A variety of pharmacological tools were used in the present study to determine if the actions of selective *μ*- and *δ*-OR agonists are mediated through a single receptor population. Transient increases in I_{sc} produced by TES in the porcine ileal mucosa have been attributed to active anion secretion, are sensitive to STX and other inhibitors of neurotransmission, and appear to be mediated by the release of several enteric transmitter substances, including acetylcholine and cyclooxygenase metabolites (Hildebrand & Brown, 1990; Poonyachoti *et al.*, 2001; Townsend IV, D. & Brown, D.R., unpublished results). As reported previously, *δ*-OR-selective agonists such as DPDPE and deltorphin II as well as the *μ*-OR-selective agonists DAMGO, endomorphin, and PL017 potently suppressed mucosal I_{sc} responses to TES (Poonyachoti *et al.*, 2001). On the other hand, the nonpeptidic *δ*-OR agonist SNC-80 exhibited a relatively low potency in suppressing tissue responses to TES, as previously reported (Poonyachoti *et al.*, 2001). In the present study, the activities of these agonists were

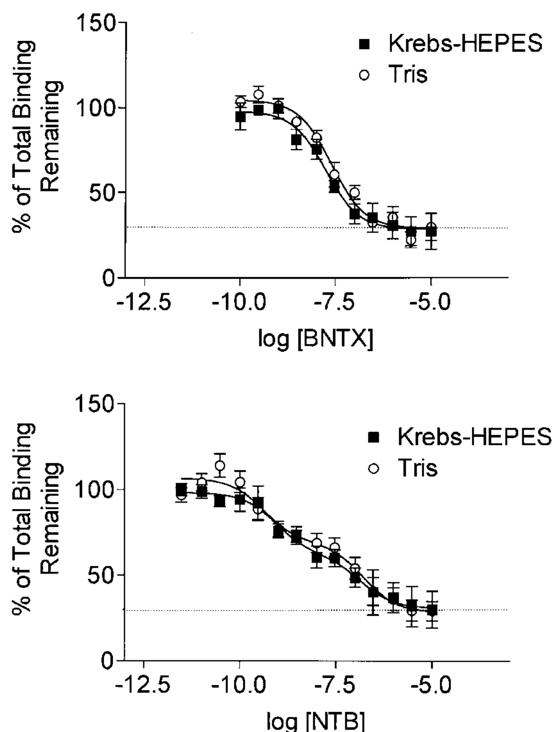

Figure 6 Displacement of [^{3}H]DPN binding in submucosal neuronal membranes by the unlabelled δ-OR antagonists BNTX (top) and naltriben (bottom). Displacement of the binding of 1 nM [^{3}H]DPN by BNTX revealed a single high-affinity binding site; the affinity of this site was not affected by changes in Na^+ concentration. Displacement with naltriben revealed two distinct binding sites whose affinities were unaffected by changes in Na^+ concentration as well. Both antagonists displaced [^{3}H]DPN to the same extent as 1 μM naloxone. Data are expressed as the percentage of total binding observed in the presence of [^{3}H]DPN alone; the abscissa indicates $\log_{10}$ molar concentration of the displacing ligand. The dashed line represents the displacement produced by 1 μM naloxone. Each point represents the mean$\pm$s.e.m. of [^{3}H]DPN binding derived from independent binding experiments in three to four assays on neuronal membranes obtained from separate pigs. The integrated results of the data analysis can be found in Table 3.

confirmed and the nonpeptidic agonist TAN-67, a selective ligand for the putative δ_1-OR, was found to be as potent and effective as its peptidic counterparts.

The potencies of selective OR antagonists at this receptor were determined previously through single dose-ratio calculations of the apparent pK_b (Gaddum, 1937). However, this analysis does not provide information on the nature of the antagonistic effect. The current study employed Schild analysis of agonist–antagonist interactions. This analysis relies on the ability of agonists to surmount the antagonism produced by reversible antagonists at varying concentrations to provide information on agonist–antagonist interactions at a common receptor (Arunlakshana & Schild, 1959). This analysis revealed that the putative δ_1-OR antagonist BNTX appeared to antagonize the antisecretory actions of DPDPE, deltorphin II, and DAMGO competitively. Its calculated pA_2 values did not differ significantly among these agonists, a result indicative of drug interactions at a single site. However, this interpertation is complicated by the fact that BNTX has affinities for both δ- and μ-ORs in the nanomolar range (Parkhill & Bidlack, 2002). Additional Schild analyses of the interactions between each of the three agonists and either naltriben or naltrindole indicated that these prototypic δ-OR antagonists do not appear to act as competitive antagonists at the OR(s) linked to ion transport in this tissue.

Nonequilibrium OR antagonists such as β-FNA or 5′-NTII appear to possess greater selectivity for particular OR types compared to their competitive counterparts, because the requirements for covalent bonding with the receptor are more stringent than interactions involving only noncovalent associations. Removal of unbound, nonequilibrium antagonist by extensively washing the tissue preparation prior to agonist addition reduces the occurrence of noncovalent interactions between these antagonists and the receptor. By effectively removing a portion of the receptor population, these receptor-alkylating drugs produce a rightward shift in the agonist concentration–effect curve, and at sufficiently high concentrations can reduce maximum agonist activity (Nickerson, 1956). The extent of this antagonism depends on two important factors, that is, the concentration of the nonequilibrium antagonist and the duration of its incubation with the receptor population. Previous studies with 5′-NTII and β-FNA have shown them to bind covalently, respectively, to δ- and μ-ORs within a 30 min exposure period at a concentration of 100 nM in isolated tissue preparations, including intestinal smooth muscle strips (Takemori *et al*., 1981; Portoghese *et al*., 1990). At concentrations up to 100-fold higher than those previously shown to block recombinant δ-ORs (Remmers *et al*., 2000), 5′-NTII failed to alter the antisecretory actions of DPDPE, deltorphin II or DAMGO in mucosa–submucosa sheets. β-FNA, at concentrations up to 10 μM, did not alter the antisecretory actions of DPDPE or deltorphin II. However, it did decrease DAMGO potency when administered at relatively high concentrations. Rightward shifts in the DAMGO concentration–effect relationship produced by β-FNA have also been documented in a guinea-pig ileum preparation, albeit at 10- to 100-fold lower concentrations than those employed in the present study (Corbett *et al*., 1985). The relatively high concentrations of β-FNA required to decrease DAMGO action might reflect the presence of spare μ-ORs in the mucosa or submucosa of porcine ileum. However, at a concentration of 1 μM, β-FNA would be expected to occupy $>$99.9% of the μ-OR population. Therefore, these μ-ORs possess either a tremendous reserve capacity, are expressed in an altered form, or are absent altogether. The low levels of [^{3}H]DAMGO binding and the absence of μ-OR-like immunoreactivity argue against the presence of a large μ-OR population (Poonyachoti *et al*., 2001; Townsend & Brown, 2002). In sum, these functional studies provide evidence for receptors exhibiting the characteristics of both μ- and δ-ORs. However, these receptors have several additional pharmacological characteristics that appear distinguish them from cloned μ- or δ-ORs. The atypical properties of selective δ-OR antagonists and the relatively low potency of SNC-80 in mucosa–submucosa sheets suggest differences from the cloned δ-OR. The low sensitivity of DAMGO action to β-FNA and the relatively low potency of morphine in this preparation indicate that they differ from the cloned μ-OR as well.

To further examine the characteristics of the receptor(s) mediating opioid action in this tissue, radioligand displacement experiments were performed using the general OR radioligand [^{3}H]DPN on neuronal membranes isolated from

Table 3 Summary of [^{3}H]DPN displacement from submucosal neuronal membranes by a variety of opioid receptor ligands

Ligand	*Receptor selectivity*	*50 mM Tris*		*Krebs–HEPES*		*IC_{50} or pK_b (nM; 95% CI)*
		K_i (nM) (*95% CI*)	*% Displaced± s.e.m. (% from high-affinity site)*	K_i (nM) (*95% CI*)	*% Displaced± s.e.m. (% from high-affinity site)*	
NTB	δ	0.077 (0.312–0.019)	78.6±12.2	0.341 (2.22–0.0522)	67.7±11.1	ND
		30.5 (113–8.3)	(52.3±7.1)	52.3 (498–5.5)	(53.1±12.8)	
SNC-80	δ	2.4 (12.3–0.5)	30.0±2.1	0.040 (0.44–0.004)	42.5±6.9	1610
				1040 (5940–182)	(30.7±8.3)	(3140–830)
TAN-67	δ	3.0 (12.3–0.74)	41.8±3.3	5.19 (13.9–1.94)	71.2±50.2	13.2
				2460 (59,900–101)	(49.4±14.3)	(82.1–2.11)
Deltorphin II	δ	3.1 (9.2–1.0)	34.0±1.7	48.6 (169–13.9)	40.5±3.2	11.3 (20.5–6.24)
BNTX	δ	4.6 (7.3–2.9)	71.0±7.3	6.6 (12.7–3.9)	69.6±8.3	134 (204–88)
DPDPE	δ	7.1 (18.1–2.8)	41.5±2.4	51.1 (142–18.3)	37.9±2.0	38.4 (74.5–19.8)
DAMGO	μ	10.7 (42.0–2.7)	74.1±12.2	119 (3660–27.5)	77.4±25.6	105
		1210 (3000–487)	(34.1±6.3)	9890 (23,900–4100)	(16.5±6.4)	(150–73.7)
Morphine	μ	71.4 (129–39.5)	65.5±6.1	643 (1570–263)	50.8±4.9	1550[a] (2570–933)

Results were obtained from membranes incubated in either Tris and Krebs–HEPES buffer. For purposes of comparison, IC_{50} or pK_b values are shown in the far-right column if known. [a]Data from Poonyachoti *et al.* (2001). N.D., not determined.

the porcine ileal submucosa. It is assumed that the receptor(s) mediating the inhibitory actions of opioid peptides on neurogenic ion transport in this tissue are represented by specific, high-affinity [^{3}H]DPN binding sites. This assumption is not unreasonable given the extensive overlap of the predicted binding domains for DPN and other oripavine-derived OR ligands, and those of a variety of structurally diverse opioid ligands, including opioid peptides and their derivatives (Pogozheva *et al.*, 1998; Filizola *et al.*, 1999). Naloxone, BNTX, the δ-OR antagonist naltriben, and the μ-OR agonists morphine and DAMGO maximally displaced approximately 70% of the total [^{3}H]DPN binding from submucous neural membranes. The displacement of [^{3}H]DPN by naltriben and DAMGO could be resolved into two distinct binding sites having a >100 fold difference in ligand affinity. We hypothesize that BNTX and morphine also displace [^{3}H]DPN from both sites, but do not have sufficient selectivity to resolve differences in binding affinities between the two sites. The higher affinity site possessed a K_i value for naltriben similar to that reported at recombinant δ-OR in cellular expression systems, whereas the lower affinity site is similar to the affinity of naltriben for the μ-OR (Raynor *et al.*, 1994; Clark *et al.*, 1997; Parkhill & Bidlack, 2002). The high-affinity DAMGO binding site determined in submucosal neuronal membranes has ≈10-fold lower affinity than those previously detected in membrane homogenates from the porcine cerebral cortex and in cultured cells expressing recombinant μ-ORs (Zastawny *et al.*, 1994; Townsend & Brown, 2002).

The highly selective δ-OR agonists deltorphin II, DPDPE, TAN-67, and SNC-80 maximally displaced only a portion of specific [^{3}H]DPN binding in submucosal neuronal membranes. Furthermore, the observation that combinations of SNC-80, DPDPE, and deltorphin II failed to produce additional displacement of [^{3}H]DPN binding, suggests that these three δ-OR agonists are binding to the same OR population. The affinity of these ligands for this binding site is not significantly different from those observed for recombinant δ-ORs (Clark *et al.*, 1997; Parkhill & Bidlack, 2002). The nature of the residual [^{3}H]DPN binding remains unclear, but it may represent a cryptic μ-OR binding site as suggested by the several lines of evidence presented above. The radioligand binding data demonstrate the presence of high-affinity binding sites for the δ-OR antagonist naltriben as well as for peptidic and nonpeptidic δ-OR agonists. It is likely that these sites represent δ-like ORs. This conclusion is supported by previous reports of δ-OR immunoreactivity in submucosal neurons from porcine ileum, detection of δ-OR mRNA transcripts in the submucosa, and the presence of high-affinity [^{3}H]naltrindole binding sites in submucous neural membranes (Brown *et al.*, 1998; Poonyachoti *et al.*, 2001; Townsend & Brown, 2002). These sites may also represent the low-affinity site observed in the DAMGO displacement curves. Although SNC-80 retains high-affinity binding to this receptor, it possesses low antisecretory potency. These findings, taken together with the results from the functional studies, suggest that the submucosal OR has pharmacological characteristics that are similar, but not identical to the cloned δ-OR.

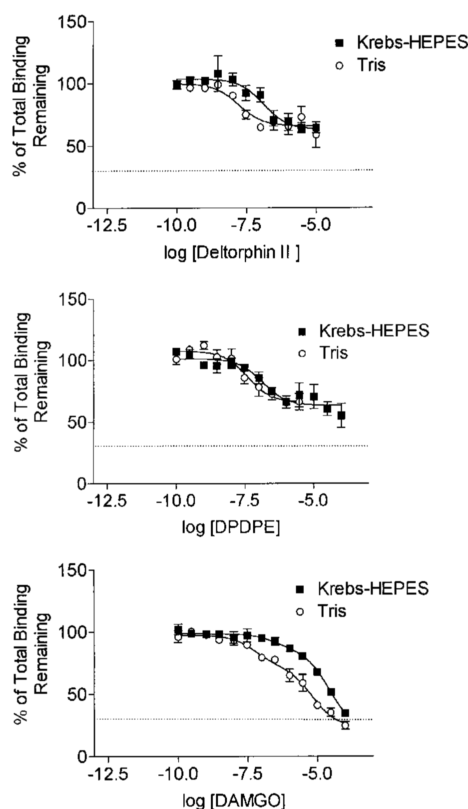

Figure 7 Displacement of 1 nM [^{3}H]DPN binding in submucosal neuronal membranes by the unlabelled δ-opioid agonists deltorphin II (top) and DPDPE (middle), or the μ-opioid agonist DAMGO (bottom). The δ-opioid agonists appear to interact with a single high-affinity binding site in membranes incubated in Na^+-free Tris buffer, and their affinity for this site is approximately five-fold lower in membranes incubated in Na^+-replete Krebs–HEPES buffer. Both ligands displaced 35–40% of total [^{3}H]DPN binding. DAMGO appears to displace [^{3}H]DPN from two binding sites in membranes incubated in Na^+-free Tris buffer, and the affinities of both sites were nine-fold lower in membranes incubated in Na^+-replete Krebs–HEPES buffer. DAMGO displaced [^{3}H]DPN to a significantly greater degree than either δ-OR agonist. Data are expressed as the percentage of total binding observed in the presence of [^{3}H]DPN alone; the abscissa indicates $\log_{10}$ molar concentration of the displacing ligand. The dashed line represents the displacement produced by 1 μM naloxone. Each point represents the mean$\pm$s.e.m. of [^{3}H]DPN binding derived from independent binding experiments in three to four assays on neuronal membranes obtained from separate pigs. The integrated results of the data analysis can be found in Table 3.

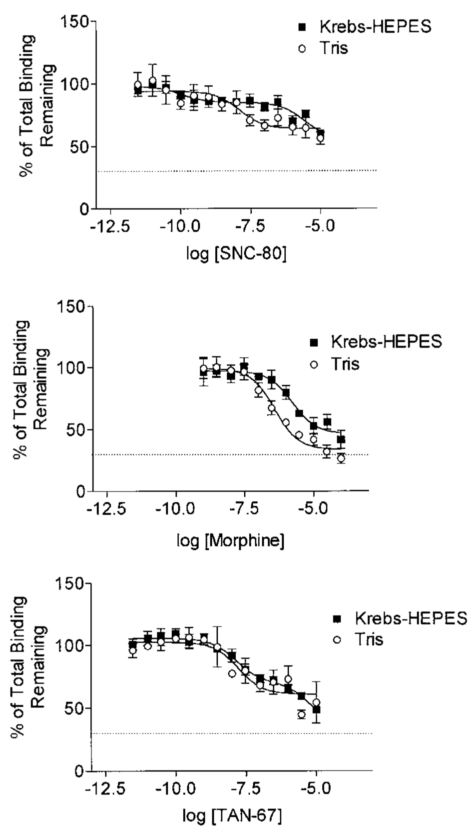

Figure 8 Displacement of 1 nM [^{3}H]DPN binding in submucosal neuronal membranes by the opioid agonists SNC-80 (top), morphine (middle), and TAN-67 (bottom). The morphine displacement curve was shifted about 10-fold to the right in membranes bathed in Na^+-replete Krebs–HEPES solution; only portions of the SNC-80 and TAN-67 displacement curves are shifted in this solution. Data are expressed as the percentage of total binding observed in the presence of [^{3}H]DPN alone; the abscissa indicates $\log_{10}$ molar concentration of the displacing ligand. The dashed line represents the displacement produced by 1 μM naloxone. Each point represents the mean$\pm$s.e.m. of [^{3}H]DPN binding derived from independent binding experiments in three to six triplicate assays on neuronal membranes obtained from three to five pigs. The integrated results of the data analysis can be found in Table 3.

Moreover, the identification of high-affinity binding sites for DAMGO in submucosal neuronal homogenates suggests that cryptic μ-ORs may also be expressed. However, as this population of specific opioid binding sites exhibits a significant difference in its affinities for morphine and DAMGO, it probably does not represent the cloned μ-OR.

The differences in the pharmacological characteristics of the submucosal OR examined in this study with those of δ- or μ-ORs from other species cannot be ascribed to large differences in porcine ORs relative to those cloned from other mammalian species. For example, the cloned porcine δ-OR possesses a deduced amino-acid sequence that is >98% identical to its cloned murine, rat, and human counterparts (Brown *et al.*, 1998), and the deduced amino-acid sequence of the porcine μ-OR is 96% identical with that of cloned human μ-OR (Pampusch *et al.*, 1998). Although we have provided evidence for the presence of ORs in the intestinal submucosa that possess pharmacological characteristics similar in some respects to μ- and δ-ORs, the existence of a novel receptor mechanism cannot be excluded. This may represent a new receptor entity; a putative δ_1-OR (Zaki *et al.*, 1996); an OR mRNA splice variant (Pasternak, 2001); or an OR heteromer, such as a δ/μ-OR heterodimer or a δ-OR homodimer (George *et al.*, 2000; Rios *et al.*, 2001). These possibilities remain to be addressed in future investigations.

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