(*green*) did not colocalize with SP immunoreactivity (*double arrows, red*). However, a few neurons may coexpress DOR and SP immunoreactivity (*vertical arrow, importing some yellowish fluorescence*). Colocalization of DOR and SP immunoreactivities was seen in nerve fibers in smooth muscle (*arrowhead, yellow*). **G, H** Sections costained with VR1 (*red*, **G, H**) and NOS (*green*, **G**) or SP (*green*, **H**) antiserum. **G** Distinct localization of VR1 and NOS immunoreactivities was observed in OSP neurons (*vertical arrow and double arrow, respectively*), and nerve fibers in circular smooth muscle (*arrowhead*). **H** Colocalization of SP and VR1 immunoreactivities was seen in some OSP neurons (*vertical arrow, yellow*), but not in nerve fibers within circular muscle (*arrowhead*) (*CM* circular muscle). *Scale bar* 200 μm

II. Study of mucosal secretory defense modulated by opioid receptor on the submucosal plexuses of porcine ileal mucosa

Effects of sensory neuropeptides or mast cell degranulator on mucosal transport

Short-circuit current (I_{sc}) and G_t attained stable baseline levels in mucosal sheets within 30 - 40 min after tissues were mounted (mean I_{sc} = -45.0 ± 5.0 μA/ cm^2 and G_t = 27.2 ± 2.0 mS/ cm^2; n = 5-10 tissues from 5-10 pigs). These parameters remained unaffected in tissues treated on their contraluminal aspect with either the neuronal conduction blocker STX (0.1 μM) or the $Na^+/K^+/Cl^-$ cotransport blocker furosemide (10 μM). The varieties of sensory neuropeptides, VR-1 agonists or mast cell degranulators compound 48/80 were added to either contraluminal or luminal sides. The carbachol increased both I_{sc} and G_t at a contraluminal concentration of 10 μM. Its effects on I_{sc} but not G_t were significantly reduced in tissues pretreated with STX or furosemide. The VR-1 agonist, capsaicin at the concentration of 1, 3 or 10 μM did not increased either I_{sc} and G_t (ΔI_{sc} = 5.0 ± 3.3 μA/ cm^2 and G_t = 30.0 ± 1.0 mS/ cm^2; n = 5-10 tissues from 5-10 pigs) (Fig.4). In contrast, sensory neuropeptides CGRP or substance P respectively increased I_{sc} from baseline to 54.65 ± 11.0 μA/ cm^2 or 34.45 ± 14.0 μA/ cm^2 but not the tissue conductance, n = 5-10 tissues from 5-10 pigs) (Fig.4). Carbachol stimulated change of both I_{sc} and G_t (ΔI_{sc} = 34.44 ± 6.7 μA/ cm^2 and G_t = 45.0 ± 3.0 mS/ cm^2; n = 5-10 tissues from 5-10 pigs) (Fig.4). To examine roles of mast cells to stimulate the secretory defense mechanism, mast cell degranulator compound 48/80 evoked both I_{sc} and G_t response to 90.20 ± 3.5 μA/ cm^2 and 35.0 ± 0.5 mS/ cm^2, respectively; n = 5-10 tissues from 5-10 pigs) (Fig.4).

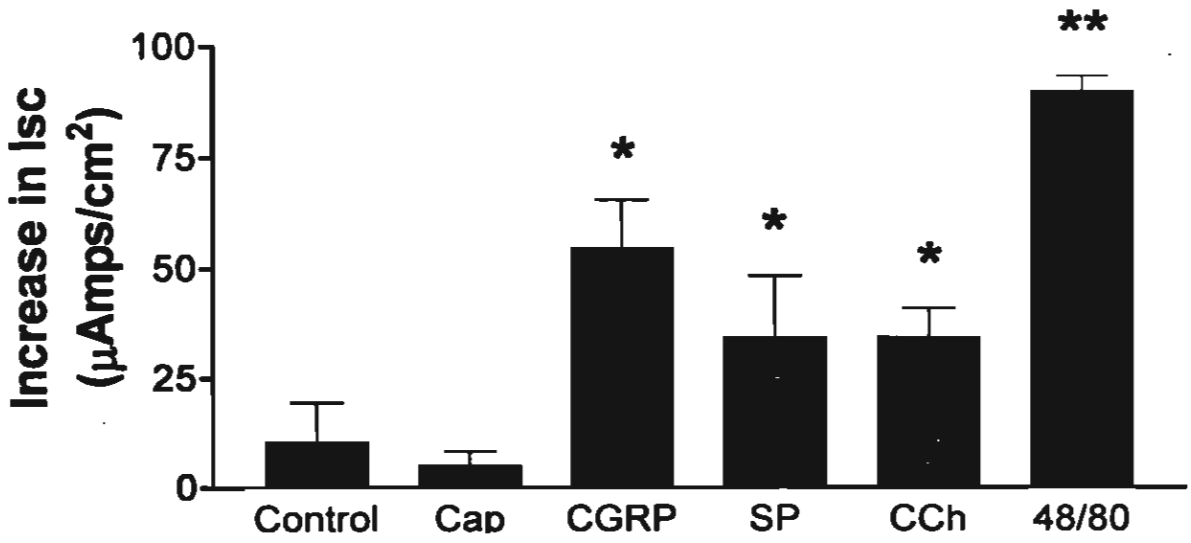

Figure 4. Histograms illustrating mucosal I_{sc} responses to contraluminal addition of vehicles (Control), vanilloid receptor type I agonist capsaicin (Cap; 3 μM), calcitonin gene-related peptides (CGRP; 100 nM), Substance P (SP; 10 μM), Carbachol (CCh;10 μM) or compound 48/80 (48/80; 10 ng/ml). Data represent the mean ± SE of drug-induced changes in I_{sc} obtained in 5 - 10 tissues from 4 - 5 pigs. Differences between mucosal responses to drugs and control are indicated as *P < 0.05 or **P < 0.01, Dunnett's t test.

Effects of blockers on sensory neuropeptides- or mast cell degranulator- evoked mucosal transport

To examine the mechanisms underlying sensory neuropeptides- or mast cell degranulator-induced increases in Isc, some tissues were pretreated contraluminally with either the neuronal conduction inhibitor saxitoxin (STX; 1 μM) or the $Na^+/K^+/Cl^-$ cotransport blocker furosemide (10 μM). STX did not significantly alter baseline I_{sc} or G_t, but it decreased mucosal I_{sc} responses to CGRP, substance P or compound 48/80 by 37.0 ± 8.0%, 84.0 ± 14.0% or 86.8 ± 6.0%, respectively; n = 5-6 tissues from 5-6 pigs) (Fig.5). STX at the same concentration had no significant effects on I_{sc} or G_t respond to carbachol (10 μM) (Fig.5).

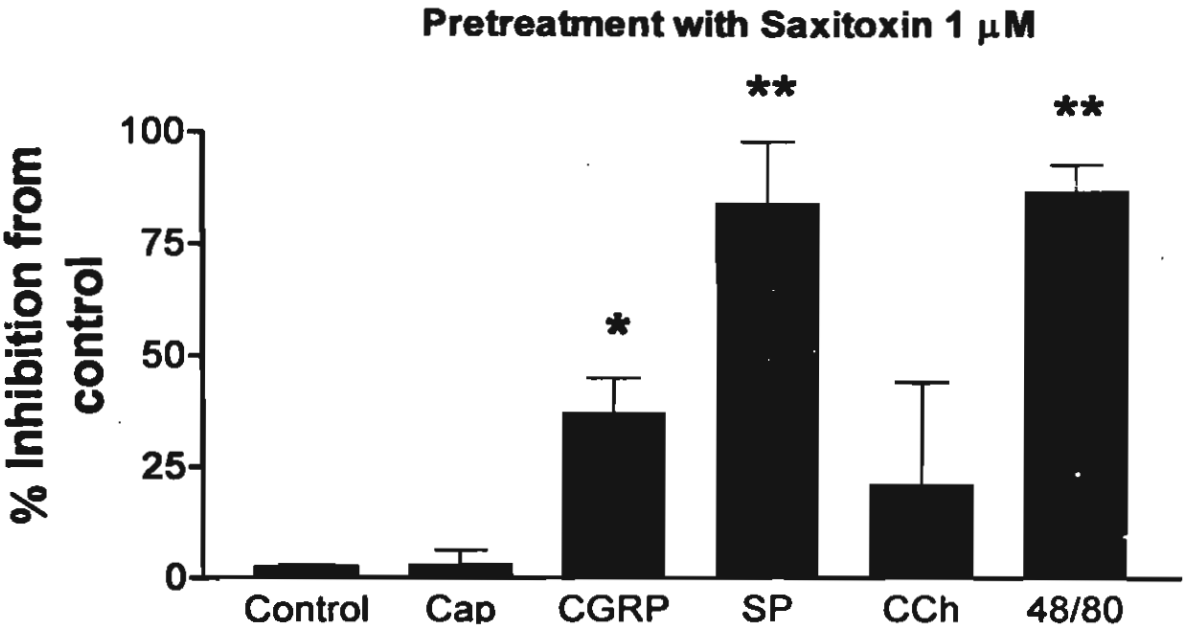

Figure 5. Histograms illustrating mucosal I_{sc} responses to vanilloid receptor type 1 agonist capsaicin (Cap; 3 μM), calcitonin gene-related peptides (CGRP; 100 nM), Substance P (SP; 10 μM), Carbachol (CCh; 10 μM) or compound 48/80 (48/80; 10 ng/ml) in absence (control) and presence of the neuronal conduction blocker saxitoxin. Five min before drugs addition, tissues were contraluminally treated with the saxitoxin at the concentration of 1 μM. Data represent the mean ± SE of drug-induced changes in I_{sc} obtained in 5 - 10 tissues from 4 - 5 pigs. Differences between mucosal responses to drugs and control are indicated as *P < 0.05 or **P < 0.01, Dunnett's t test.

The secretion of chloride ion underlying the actions of sensory neuropeptides- or mast cell-evoked changes of I_{sc} were further investigated. the contraluminally addition of furosemide (10 μM) significantly inhibited I_{sc} responses to CGRP, substance P, carbachol or compound 48/80 by 75.10 ± 10.0%, 89.3 ± 30.0%, 90.0 ± 1.0% or 96.3 ± 12.0%, respectively; n = 5-10 tissues from 5-10 pigs) (Fig.6). In addition, baseline I_{sc} or G_t was not altered by pretreatment tissues with furosemide at the same concentration (control, Fig.6).

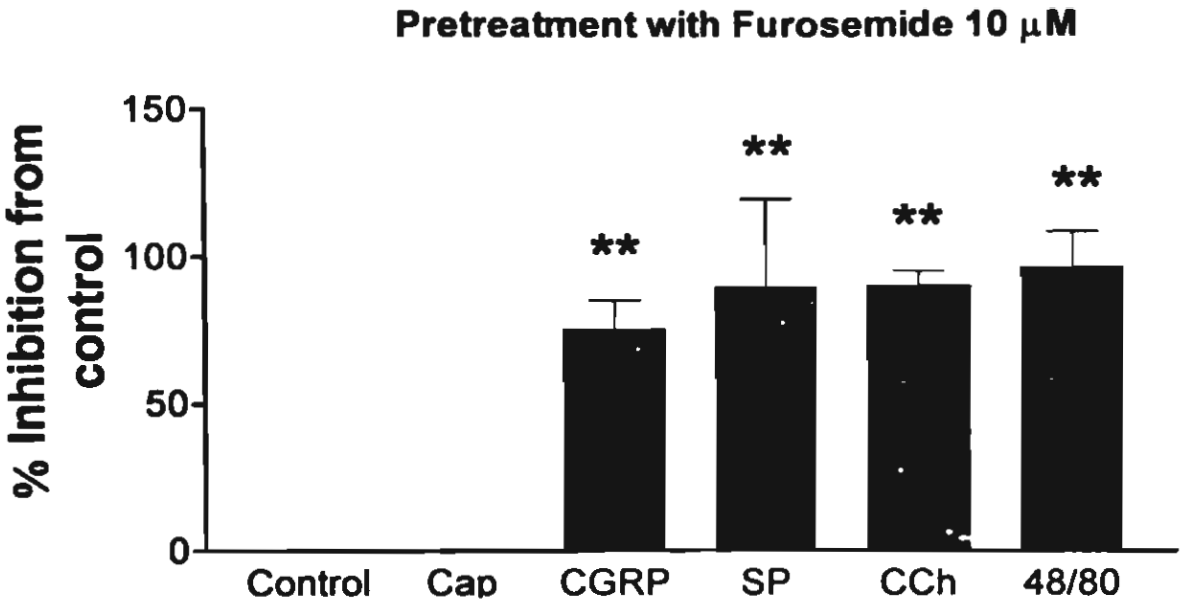

Figure 6. Histograms illustrating mucosal I_{sc} responses to vanilloid receptor type I agonist capsaicin (Cap; 3 μM), calcitonin gene-related peptides (CGRP; 100 nM), Substance P (SP; 10 μM), Carbachol (CCh;10 μM) or compound 48/80 (48/80; 10 ng/ml) in absence (control) and presence of the $Na^+/K^+/Cl^-$ cotransport blocker furosemide. Five min before drugs addition, tissues were contraluminally treated with the furosemide at the concentration of 10 μM. Data represent the mean ± SE of drug-induced changes in I_{sc} obtained in 5 - 10 tissues from 4 - 5 pigs. Differences between mucosal responses to drugs and control are indicated as **P < 0.01, Dunnett's t test.

Effects of delta-opioid receptor agonist on neuropeptide-induced mucosal transport

The selective δ-OR agonist DPDPE, at a contraluminal concentration of 0.1 μM, had no significant effect on either baseline or stimulation of I_{sc} by capsaicin or carbchol (Fig. 7). The inhibitory effects of DPDPE on CGRP-, SP- or compound 48/80-induced ΔI_{sc} by 25.1 ± 10.0%, 76.5 ± 21.0% or 49.6 ± 14.0%, respectively; n = 5-10 tissues from 5-10 pigs) (Fig.7). In addition, DPDPE had no effect on G_t of 48/80-induced increases in G_t. The inhibitory effects of DPDPE were reversed in tissues pretreated with the selective OR antagonist naloxone 0.1 μM. Naloxone alone did not significantly alter mucosal electrical parameters. In the presence of STX, DPDPE had no significant additional inhibitory effect on CGRP-, SP- or 48/80-induced elevations in I_{sc} (n = 3 tissues from 3 pigs).

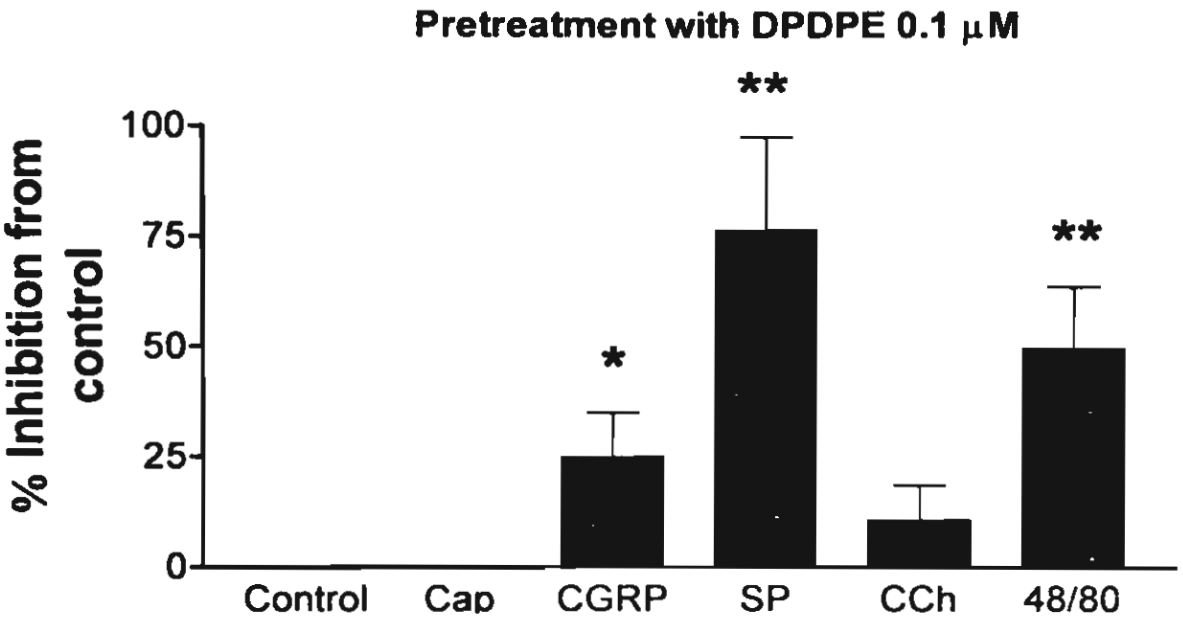

Figure 7. Histograms illustrating mucosal I_{sc} responses to vanilloid receptor type I agonist capsaicin (Cap: 3 μM), calcitonin gene-related peptides (CGRP: 100 nM), Substance P (SP; 10 μM), Carbachol (CCh:10 μM) or compound 48/80 (48/80: 10 ng/ml) in absence (control) and presence of the selective δ-OR agonist DPDPE. Five min before drugs addition, tissues were contraluminally treated with the DPDPE at the concentration of 0.1 μM. Data represent the mean ± SE of drug-induced changes in I_{sc} obtained in 5 - 10 tissues from 4 - 5 pigs. Differences between mucosal responses to drugs and control are indicated as *P < 0.05 or **P < 0.01. Dunnett's t test.

Discussion and conclusion

As previously reported, DOR immunoreactivity was localized in neurons and fibers of all enteric plexuses within the wall of the porcine ileum (Brown et al. *1998*). In contrast, immunoreactivity to KOR was confined to the myenteric plexus. A predominant localization of KOR immunoreactivity in MP neurons and nerve fibers has also been reported in the rat small intestine and colon (Bagnol et al. *1997*). Immunoreactivity to DOR appeared to be expressed predominately in ChAT-positive enteric neurons suggesting DOR and KOR in the MP may modulate intestinal motor function. Functional studies in a circular muscle-MP preparation from porcine ileum have shown that stimulation of DOR or KOR with receptor-selective agonists is associated with an inhibition of neurogenic contractions (Brown et al. *1998*; Poonyachoti et al. *2001a*). In agreement with the presence of DOR immunoreactivity, but not KOR in the submucosal neurons, it has recently been reported that DOR-selective agonists potently suppress neurogenic anion transport in porcine ileal mucosa containing an intact ISP; KOR-selective agonists were nearly 1000-fold less potent in this preparation (Poonyachoti et al. *2001b*). There is no functional or immunohistochemical evidence for the expression of μ-ORs in porcine ileum (Poonyachoti et al. *2001a, 2001b*). This stands in contrast to the rat and guinea pig small intestine in which μ-OR immunoreactivity was observed to predominate in enteric neurons and nerve fibers (Bagnol et al. *1997*; McConalogue et al. *1999*), and probably represents a major species difference in OR expression. Immunoreactivity to one class of endogenous OR ligands, the enkephalins, are prominent in neurons and fibers of the porcine MP, but are relatively sparse in the submucosal plexuses (Porcher et al. *2000*). These opioid peptides are therefore likely to activate myenteric ORs under physiological conditions. It is possible that another class of endogenous ligands interacts with DOR expressed in the ISP, a receptor with pharmacological characteristics that are quite different from DOR in the MP (Poonyachoti et al. *2001b*).

VR1 immunoreactivity, like that to DOR, was distributed in all three ganglionated plexuses of porcine ileum, where it was also highly colocalized in ChAT-immunoreactive neurons. Its expression in the perikarya and fibers of enteric neurons is similar to its immunocytochemical pattern of expression in dorsal root ganglion neurons (Guo et al. *1999*).

Immunoreactivity to DOR or VR1 was frequently colocalized in enteric neurons displaying immunoreactivity to the sensory neuropeptide CGRP. Immunoreactivity to DOR has been colocalized with CGRP immunoreactivity in dorsal root ganglion neurons and fibers (Dado et al. *1993*; Guo et al. *1999*). Moreover, KOR-

and DOR-positive dorsal root ganglion neurons are sensitive to the VR1 agonist capsaicin (Zhang et al. *1998*) and activation of KOR decreases CGRP release into the intrathecal space (Collin et al. *1993*). VR1 and CGRP immunoreactivities are highly colocalized in trigeminal ganglia (Ichikawa and Sugimoto *2001*), and the VR1 agonists capsaicin and anandamide evoke CGRP release from nerve terminals in the dorsal spinal cord (Tognetto et al. *2001*). In vascularly perfused, isolated segments of porcine ileum, capsaicin infusion is associated with a marked increase in CGRP release (Rasmussen et al. *2001*). Therefore, ChAT-/CGRP-positive enteric neurons that coexpress OR and VR1 immunoreactivities may represent intrinsic primary afferent neurons (IPANs) which coordinate enteric secretomotor and vasodilatory reflexes (Furness et al. *1998*). In rat and guinea pig small intestine, IPANs manifest ChAT immunoreactivity (Li and Furness *1998*; Mann et al. *1999*) as do CGRP-immunoreactive Dogiel type 2 neurons in the porcine small intestine (Scheuermann et al. *1987*; Hens et al. *2000*). Immunoreactivity to another putative sensory transmitter, SP, small populations of SP-positive submucosal neurons were immunoreactive for either DOR or VR1; these latter neurons may have a secretomotor role in the porcine intestine (Hens et al. *2000*). The functional roles served by VR1-immunoreactive neurons in the enteric nervous system were investigated in the present study.

In the porcine small intestine, the OSP have considerably fewer mucosal projections than the ISP (Hens et al. *2000*). Neurons in these plexuses innervate the intestinal smooth muscle layers and send viscerofugal projections to the prevertebral ganglia (Timmermans et al. *1997*). In the present study, there was substantial overlap between VR1 and SP immunoreactivities in OSP neurons. These results indicate that there are some differences in the receptor signatures of chemically coded neurons in these two ganglionated plexuses.

NOS-immunoreactive neurons have previously been reported to predominate in the OSP and MP of porcine small intestine (Timmermans et al. *1994*). Their presence in the ISP neurons observed in the present study may reflect segmental differences in NOS expression along the length of the intestine. Neurons coexpressing opioid and vanilloid receptors that are situated in OSP may modulate intestinal segmentation and propulsion. Moreover, DOR and VR1 immunoreactivities also could be expressed by IPANs in the OSP that sense luminal chemicals, mucosal distortion or intestinal distension and evoke mechanical reflexes to assist the digestive process or speed the elimination of luminal pathogens.

Nearly 80% of neurons with axonal projections to the intestinal mucosa reside in the ISP of porcine ileum; about half of these include distinct subpopulations of

ChAT-immunoreactive neurons (Hens et al. *2000*). DOR and VR1 immunoreactivities may be localized on one or more subsets of ChAT-positive, VIP-negative ISP neurons with mucosal projections that have been reported in porcine small intestine; in addition to large type 2 sensory neurons with a ChAT/CGRP/(SP) phenotype, these neurons include a major subpopulation of small ChAT/SP-immunoreactive neurons and a very small population of multidendritic ChAT/somatostatin-immunoreactive neurons which have putative secretomotor roles (Hens et al. *2000*).

We recently investigated that DOR modulates a neural pathway in the porcine ileal submucosa that links intestinal inflammation and hypersensitivity with transepithelial secretion, an important mucosal defense mechanism (Perdue and MacKay *1994*). DOR and VR1 immunoreactivities are expressed in CGRP-positive ISP neurons, and there is increasing evidence that imbalances in the enteric neural circuits containing sensory transmitters such as CGRP are involved in the pathophysiological alterations of mucosal secretion, blood flow and immune function that are associated with intestinal infection, allergy and inflammation (Holzer *1998*). Neurons in the ISP coexpressing CGRP, VR1 and DOR may play an important role in sensing and modulating these pathophysiological changes in mucosal function. In the guinea pig ileum for example, the VR1 agonist capsaicin induces active anion secretion and increases submucosal blood flow (Vanner and MacNaughton *1995*; Vanner and Bolton *1996*). Enteric neurons expressing opioid and vanilloid receptors may constitute important therapeutic targets for the development of drugs alleviating painful intestinal inflammatory, allergic and dysmotility states. Drugs interacting with DOR or VR1 may be useful in alleviating some of the manifestations of inflammatory bowel disease. Abdominal pain is associated with inflammation, anaphylaxis or ischemia and submucosal nerves that express DOR may mediate the analgesic actions of opioids in these conditions (Bueno et al. *2000*).

The evidences of their colocalizations. DOR-/CGRP- or DOR-/VR-1-immunoreactive neurons in the submucosal may include primary afferents and constitute novel therapeutic targets for the palliation of painful intestinal inflammatory and hypersensitivity. Generally, stimulation of the VR-1 with its agonist the capsaicin results in the activation of nociceptive or releasing of SP or CGRP from enteric neurons, and stimulated anion secretion in the rodent intestines. However, in the recent study of ion transport measurement in the porcine ileal mucosa, capsaicin (1-10 μM) could not alter the change of Isc or Gt from the baseline. In contrast, CGRP or SP potently increased change of Isc from baseline to 30-50 $\mu A/cm^2$ which are insensitive to saxitoxin or furosemide. The recent results in the porcine mucosa argued the previous studies that the stimulation effect of capsaicin on the releasing of SP or CGRP from the sensory neurons and induced the neurogenic anion secretions. The evidences may imply the different

roles of IPANs on the neurogenic ion secretion between the different species. The compound 48/80 which degranulated mast cell to simulate the intestinal inflammatory phenomena also increased Isc to 80 $\mu A/cm^2$. Even though its effect was higher than those of SP or CGRP, it was also blocked by STX or furosemide. The potent results of compound 48/80 suggest that inflammatory mediators. i.e. histamine, serotonin released from mast cells play an important role to exert neurogenic active ion secretion. Opioids were hypothesized to inhibit neurotransmitter released and cause the inhibiting effects on the secretorymotor reflex via opioid receptors presented on the enteric neurons. In this study, selective *delta*-opioid agonist DPDPE 10 μM inhibited active anion secretion-stimulated by SP, CGRP or compound 48/80 by 75%. 25% or 50%. respectively. These evidences were reversed by naloxone 1 μM suggesting the neuronal OR mediated the inhibitory effects of DPDPE. The evidences implied DOR expressed on neurons including the IPANs in submucosal plexuses plays roles in inhibit neurogenic anion secretion evoked by IPANs transmitters or mast cell degranulation. DOR and its agonists may have effects on the other intestinal function. i.e. nociceptive. immunity. motility regulated by IPANs or mast cell-mediated inflammation. Impairments in host defense processes of the intestine due to abuse of opioid or in the other illicit. neuroactive drugs could set the stage for the initiation and systemic spread of bacteria and viral infection. As a major neuroimmune locus, the intestinal mucosa is an important and clinically-relevant system for investigating the actions of neuroactive drugs on specific and non-specific host defense processes and for the identification of novel therapeutic strategies to enhance mucosal immunity and combat microbial interactions. Therefore. the relationships between IPANs and neuronal DOR need to be further studied in order to be the novel intestinal drug targets in human.

Future Directions

1. To further study the compound 48/80 with the selective blockers of neurochemical drugs.
2. To further study the CGRP, substance P and compound 48/80 in modulating mast cell and immunocyte function in the small intestine.
3. To further study DOR in modulating neuropeptide- or neurochemical-induced mast cell and immunocyte function in the small intestine
4. To further study DOR vs. neuropeptides- or neurochemical drugs on the protection of bacterial or infection disease in small intestine.

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REGULAR ARTICLE

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Chemical coding of neurons expressing δ- and κ-opioid receptor and type I vanilloid receptor immunoreactivities in the porcine ileum

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Abstract Opioid drugs have profound antidiarrheal and constipating actions in the intestinal tract and are effective in mitigating abdominal pain. Mediators of intestinal inflammation and allergy produce increased mucosal secretion, altered bowel motility and pain due to their ability to evoke enteric secretomotor reflexes through primary afferent neurons. In this study, the distribution of δ- and κ-opioid receptor (DOR and KOR, respectively) immunoreactivities in chemically identified neurons of the porcine ileum was compared with that of the capsaicin-sensitive type 1 vanilloid receptor (VR1). DOR and VR1 immunoreactivities were observed to be highly localized in choline acetyltransferase (ChAT)- and calcitonin gene-related peptide (CGRP)-positive neurons and nerve fibers of the submucosal and myenteric plexuses and both receptors exhibited frequent colocalization. In the inner submucosal plexus, they also were colocalized in substance P (SP)-positive neurons. Neurons in the outer submucosal plexus expressed DOR immunoreactivity alone or in combination with VR1. KOR-immunoreactive neurons were found only in the myenteric plexus; these cells coexpressed immunoreactivity to ChAT, CGRP, vasoactive intestinal peptide (VIP) or nitric oxide synthase (NOS). In addition, some KOR-positive neurons coexpressed immunoreactivities to DOR and VR1. Based on their neurochemical coding, opioid and vanilloid receptor-immunoreactive neurons in the submucosal and myenteric plexuses may include primary afferents and constitute novel therapeutic targets for the palliation of painful intestinal inflammatory, hypersensitivity and dysmotility states.

Keywords Choline acetyltransferase · Calcitonin gene-related peptide · Substance P · epithelial ion transport · Intestinal propulsion · Sensory neurotransmission · Pig (Yorkshire)

Introduction

For centuries, opium has been used for the alleviation of pain and palliation of diarrheal disease. The pharmacological effects of botanically derived opiates, endogenous opioid peptides, and their synthetic analogs on gastrointestinal motility and mucosal transport are well known (De Luca and Coupar 1996). Their actions on the small intestine include decreases in propulsive motor activity, the prolongation of intestinal transit time and inhibition of the neurogenic secretion of anions and water. The antimotility and antisecretory effects of opioids are mediated through the enteric and central nervous systems. By interacting with opioid receptors (ORs) on enteric neurons, opioids can alter the release of both excitatory and inhibitory enteric neurotransmitters (De Luca and Coupar 1996). Although immunoreactivities to opioid peptides, such as the enkephalins or dynorphin, have been localized in enteric neurons, little is known about the distribution of their cognate receptors in the enteric nervous system.

Capsaicin, a lipophilic vanilloid substance found in *Capsicum* peppers which produces the sensation of heat, has a prominent excitatory action on primary afferent neurons. This neuronal action, which is mediated through type 1 vanilloid receptors (VR1) coupled to a non-selective cation conductance, is associated with nociception and localized release of proinflammatory substances (Szallasi and Blumberg 1999). Protons, generated under conditions of tissue ischemia or inflammation, also modulate VR1 activity. When administered in the intestinal lumen of humans, capsaicin evokes sensations

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Table 1 Description of antibodies used for immunohistochemistry in the present study (*ChAT* choline acetyltransferase, *CGRP* calcitonin gene-related peptide, *DOR* δ-opioid receptor, *KOR* κ-opioid receptor, *NOS* neuronal nitric oxide synthase, *PGP 9.5* protein gene product 9.5, *SP* substance P, *VIP* vasoactive intestinal peptide, *VR1* type 1 vanilloid receptor)

Antigen	Host	Dilution	Source
ChAT	Goat	1:20	Chemicon International Inc., Temecula, CA
	Rabbit	1:400	Chemicon International Inc.
CGRP	Goat	1:400	Santa Cruz Biotechnology, Santa Cruz, CA
	Rabbit	1:400	Chemicon International Inc.
DOR 461	Rabbit	1:400	Dr. Robert P. Elde, University of Minnesota
KOR-1	Goat	1:400	Santa Cruz Biotechnology
NOS	Rabbit	1:600	Santa Cruz Biotechnology
PGP 9.5	Rabbit	1:400	Chemicon International Inc.
SP	Rat	1:40	Incstar, Stillwater, MN
VIP	Rabbit	1:600	Incstar
VR1	Guinea pig	1:400	Dr. Robert P. Elde, University of Minnesota

of abdominal pain (Hammer et al. 1998). Recent immunocytochemical investigations using novel antisera directed against unique peptide sequences in the amino- and carboxy-termini of VR1 have demonstrated that receptor-like immunoreactivity is expressed in the perikarya and terminals of neurons, where it is colocalized with binding sites for the lectin IB4 and immunoreactivity to $P2X_3$ purinoceptors (Guo et al. 1999). VR1-immunoreactive neurons have also been detected in several brain locations (Mezey et al. 2000). Mice deficient in the VR1 gene manifest impairments in nociception and pain-related behaviors, and dorsal root ganglion neurons from these animals lack VR1 immunoreactivity, but not IB4 binding sites, and do not respond with changes in membrane potential to vanilloids, protons or heat (Caterina et al. 2000).

Recent immunohistochemical studies have demonstrated the presence and distribution of OR immunoreactivity in the rat and swine enteric nervous systems (Bagnol et al. 1997; Brown et al. 1998). In the porcine ileal mucosa, δ-OR (DOR) -immunoreactive nerve fibers lie in close proximity to mucosal mast cells and the highly selective DOR agonist [D-Pen^{2,5}]enkephalin suppresses neurogenic secretion induced by histamine, the mast cell degranulator compound 48/80, and the food allergen β-lactoglobulin (Poonyachoti and Brown 2001). Furthermore, [D-Pen^{2,5}]enkephalin inhibits neurogenic secretion in response to trypsin, an indirect agonist at type 2 proteinase-activated receptors and a model for the action of mast cell tryptase (Green et al. 2000). Substance P (SP)- and calcitonin gene-related peptide (CGRP)-containing primary afferent neurons in the spinal cord have been found to coexpress immunoreactivity to these receptors (Steinhoff et al. 2000). These results suggest a common enteric neural pathway may mediate intestinal inflammation and opioid antisecretory activity. Therefore, in this study we examined the chemical coding of enteric neurons expressing immunoreactivity to DOR and κ-OR (KOR) in the porcine ileum and the relationship of OR immunoreactivity to that of VR1. Our experiments suggest that ORs and VR1 coexist on cholinergic neurons within all enteric ganglia of the porcine small intestine and implicate these receptors in the modulation of intestinal sensorimotor function.

Materials and methods

Five Yorkshire pigs (6–10 weeks old) of each sex were obtained from the University of Minnesota Swine facility (Rosemount, MN). They were provided with standard feed (Rosemount Feed Mill, Rosemount, MN) ad libitum. Animals were anesthetized by intramuscular injection of a tiletamine hydrochloride-zolazepam combination (Telazol; 8 mg/kg, Fort Dodge Laboratories, Fort Dodge, IA), in combination with xylazine (8 mg/kg), and subsequently euthanized by barbiturate overdose in accordance with approved University of Minnesota Animal Care Committee protocols. Tissues were obtained and processed as described previously (Kulkarni-Narla et al. 1999).

DOR and KOR immunoreactivities were detected with a rabbit polyclonal antiserum against an extracellular sequence (PFQSAKYLMETWPFGELL) of the mouse DOR that is conserved in porcine DOR and generously provided by Dr. Robert P. Elde (Arvidsson et al. 1995; Brown et al. 1998), and a goat polyclonal antiserum raised against the N-terminus of the human KOR (MESPIQIFRGEPGPTCAPSA) purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). VR1 immunoreactivity was detected using a polyclonal antiserum raised in guinea pig against the C-terminus of murine VR1 that was provided by Dr. Robert P. Elde (Guo et al. 1999). Anticholine acetyltransferase (ChAT) and antineuronal nitric oxide synthase (NOS) antisera were used to detect cholinergic and nitrinergic neural elements, respectively. Antisera to the vasoactive intestinal peptide (VIP), CGRP and SP were employed to investigate the peptidergic coding of enteric neurons. To confirm neuronal morphology, adjacent sections to each antisera-stained section were incubated with an antibody to the neuronal marker, protein gene product 9.5 (PGP 9.5). Details of the antibodies employed are given in Table 1.

To investigate colocalization of the various markers, coincubation of tissue sections with DOR or KOR antiserum alone and in combination with other primary antibodies were performed. In addition, some tissue sections were coincubated simultaneously with DOR, KOR and VR1 antisera. The neurochemical coding of OR- and VR1-immunoreactive neurons and fibers was examined with antibodies to ChAT, VIP, CGRP, SP and NOS. Because DOR, NOS and VIP antisera were raised in rabbits, neurons coexpressing immunoreactivities to DOR and ChAT were compared with those coimmunostained with ChAT antisera and NOS or VIP antisera in adjacent or nearby sections.

Tissue sections were rehydrated in phosphate-buffered saline (PBS, pH 7.4) for 15 min and incubated in PBS containing 0.4% Triton X-100 (Triton; Sigma Chemical Co., St. Louis, MO) and 3% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature to block non-specific binding of the primary antibodies. Sections were further incubated overnight at 4°C with primary antisera diluted in Triton/BSA-containing PBS. Following rinses in PBS for 15 min, sections were incubated in PBS for 1 h in the dark with one or more of the following secondary antibodies: donkey anti-rabbit, goat or rat indocarbocyanine 3 (Cy3)-conjugated IgG at 1:400 dilution; donkey anti-goat, anti-rat or anti-rabbit fluorescein isothiocyanate (FITC)-

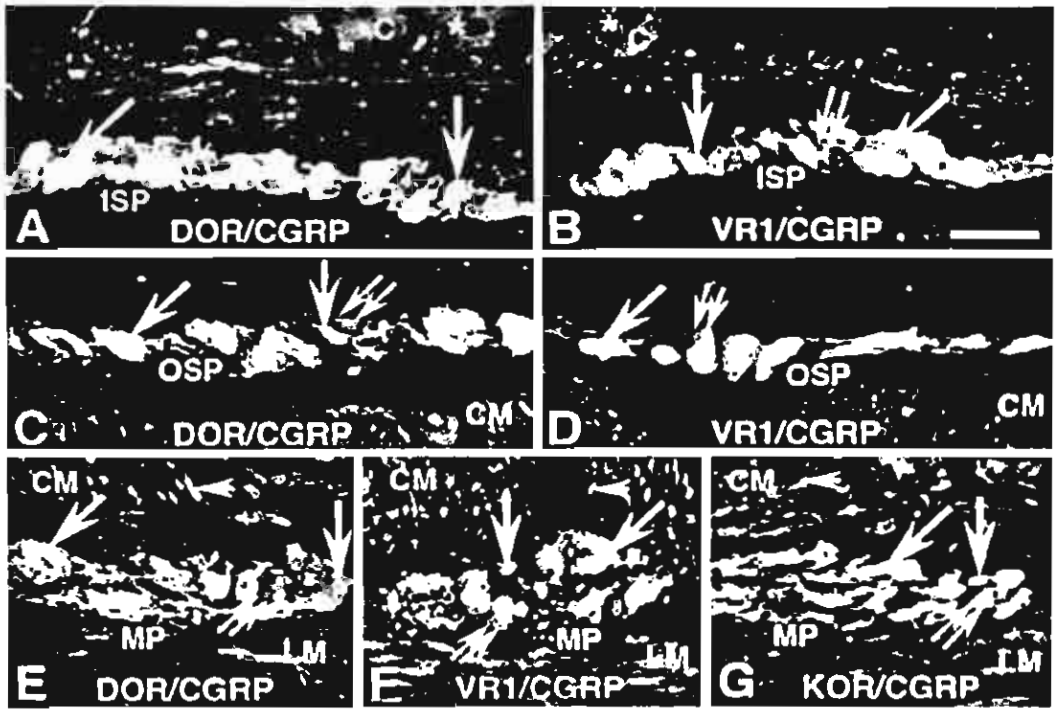

Fig. 1A–G Photomicrographs of representative longitudinal sections of porcine ileum showing colocalization of δ-opioid receptor (*DOR*), κ-opioid receptor (*KOR*) or type 1 vanilloid receptor (*VR1*) immunoreactivity in calcitonin gene-related peptide (*CGRP*)-immunoreactive neurons in the porcine enteric nervous system. **A, B** Colocalization (*yellow*) of DOR (*green*, A) or VR1 (*green*, B) immunoreactivity with CGRP (*red*) immunoreactivity was observed in neurons (*slanted arrows*) in the inner submucosal plexus (*ISP*). Some DOR- or VR1-immunoreactive neurons (*vertical arrows*) did not express CGRP immunoreactivity. **C, D** Colocalization (*yellow*) of DOR (*green*, C) or VR1 (*green*, D) immunoreactivity with CGRP (*red*) immunoreactivity was observed in neurons (*slanted arrows*) in the outer submucosal plexus (*OSP*). Some DOR- or VR1-immunoreactive neurons (*vertical arrows*) did not express CGRP immunoreactivity. **E–G** Colocalization (*yellow*) of DOR (*green*, E), VR1 (*green*, F) or KOR (*green*, G) immunoreactivity with CGRP (*red*) immunoreactivity was observed in neurons (*slanted arrows*) in the myenteric plexus (*MP*). *Asterisks* denote non-specific staining seen on or under the epithelium in the section that persisted in omission and preabsorption control experiments (*C* crypt, *CM* circular muscle, *LM* longitudinal muscle). *Scale bar* 60 μm (A–D), 100 μm (E–G)

conjugated IgG at 1:40 dilution; or donkey anti-rabbit indocarbocyanine 5 (Cy5). Sections were subsequently washed in PBS for 15 min and coverslipped with Vectashield (Vector Laboratory, Burlingame, CA).

Control experiments to verify the specificity of primary antibodies were performed by preincubating primary antibodies with their corresponding blocking peptides in 10- to 30-fold excess by weight. Blocking peptides were preincubated overnight at 4°C with primary antiserum at the same dilutions used to detect immunoreactive structures in situ; after centrifugation, the supernatant obtained was substituted for the primary antibody in the staining protocol. Preincubation of DOR, KOR or VR1 antisera with their respective blocking peptides in 30-fold excess eliminated specific neuronal immunoreactivity, but not immunofluorescence in the crypts and muscularis mucosae. For all other primary antibodies, incubation of sections with blocking peptides in tenfold excess abolished specific immunoreactivity. Omission of primary antibodies resulted in an absence of specific immunoreactivity.

A minimum of three transverse or longitudinal ileal sections from each experiment were scanned using a BioRAD confocal laser scanning microscope (Model 1024) which was attached to a Nikon fluorescence microscope. Each field was scanned sequentially in three dimensions by optical sectioning with a step size of 0.5 μm. Images were obtained using Comos software (version 6.05.8; Comos BioRad, Hercules, CA) and further processed employing NIH Image (version 1.59) and Adobe Photoshop (version 4.0, Adobe Systems, San Jose, CA). The numbers of neurons that exhibited colocalization of opioid or vanilloid receptor immunoreactivities with that of other neurochemical markers were counted in inner and outer submucosal ganglia and myenteric ganglia in two different fields from each of three pigs. Data are expressed as the mean ± SD of the percentage of the total number of immunoreactive neurons counted for each pair of antigens examined.

Results

DOR- and VR1-immunoreactive neurons and fibers were observed in all enteric plexuses in porcine ileum. In comparison, immunoreactivity to KOR was expressed only in the myenteric plexus and nerve fibers innervating circular smooth muscle. All structures exhibiting specific immunoreactivity to receptors and neurochemicals expressed PGP 9.5 immunoreactivity in adjacent tissue sections. Immunoreactivity to CGRP, a neurochemical marker of putative Dogiel type 2 sensory neurons in the porcine small intestine (Scheuermann et al. 1987), was highly expressed in several DOR- and VR1-immunoreactive neurons in the inner and outer submucosal plexuses (Fig. 1A–D, Table 2). It was colocalized with DOR, KOR and VR1 immunoreactivities in myenteric neurons as well (Fig. 1E–G, Table 2).

Table 2 Occurrence of immunoreactive neurons in enteric ganglia. Data represent the mean ± SD of the percentage of total immunoreactive neurons counted for each pair of antigens examined; neurons in submucosal or myenteric ganglia were counted in a total of six different microscopic fields in tissue sections obtained from three pigs. Abbreviations are the same as in Table 1

Antigens	Total number of immunoreactive neurons	% Co-immunoreactive neurons	% Neurons immunoreactive for first antigen only	% Neurons immunoreactive for second antigen only
Inner submucosal ganglia				
DOR/ChAT	119	81±5	5±4	14±5
DOR/CGRP	117	80±7	10±5	10±3
DOR/SP	113	22±8	77±8	1±2
DOR/VR1	125	83±5	5±5	13±3
VR1/ChAT	119	70±8	12±4	18±8
VR1/CGRP	125	82±5	12±3	6±4
VR1/SP	117	12±6	67±11	21±8
Outer submucosal ganglia				
DOR/ChAT	70	64±15	3±4	33±13
DOR/CGRP	77	77±13	9±8	15±6
DOR/SP	71	0±0	70±8	30±8
DOR/VR1	68	59±12	3±5	37±11
VR1/ChAT	67	94±6	2±4	4±6
VR1/CGRP	69	89±11	3±4	8±7
VR1/SP	64	39±8	43±8	17±3
Myenteric ganglia				
DOR/ChAT	146	71±8	19±6	10±2
DOR/CGRP	129	69±14	9±3	22±14
DOR/SP	144	0±0	77±7	23±7
DOR/VR1	134	79±4	12±4	9±3
VR1/ChAT	135	88±8	7±5	4±5
VR1/CGRP	138	73±8	5±3	22±6
VR1/SP	133	0±0	82±9	19±7
KOR/ChAT	144	44±7	10±4	46±7
KOR/NOS	130	10±3	70±7	20±5
KOR/VIP	136	46±12	11±5	43±6
KOR/CGRP	129	72±11	9±5	18±7
KOR/SP	139	0±0	78±11	23±9

Inner submucosal plexus

DOR immunoreactivity was highly colocalized with ChAT immunoreactivity in inner submucosal plexus (ISP) neurons at the base of crypts (Fig. 2A). Table 2 indicates the relative percentages of immunoreactive neurons counted that exhibited colocalization of receptor immunoreactivities with those of other neurochemical markers. Immunoreactivity to NOS (Fig. 2B) or VIP (Fig. 2C) was absent in ChAT-positive neurons which were immunoreactive to DOR in adjacent sections. ChAT and DOR immunoreactivities were also colocalized with VR1 immunoreactivity in ISP neurons (Fig. 2D, E, Table 2). Subpopulations of DOR- and VR1-positive neurons also expressed immunoreactivity to SP (Fig. 2F, H, Table 2). VR1-immunoreactive ISP neurons did not express NOS immunoreactivity (Fig. 2G).

Outer submucosal plexus

DOR- and VR1-immunoreactive neurons were observed in the outer submucosal plexus (OSP); these neurons coexpressed ChAT immunoreactivity (Fig. 3A, D, Table 2). Some ChAT-positive neurons which were also DOR-positive, coexpressed NOS (Fig. 3B) or VIP immunoreactivity (Fig. 3C) in adjacent sections. There was substantial colocalization of DOR and VR1 immunoreactivities, although some neurons expressing VR1 immunoreactivity alone were observed (Fig. 3E, Table 2). There was sparse colocalization of DOR and SP immunoreactivities in neurons (Fig. 3F), but there was moderate colocalization of VR1 and SP immunoreactivities in OSP neurons (Fig. 3H, Table 2). VR1-immunoreactive neurons did not express NOS immunoreactivity; however, NOS-immunoreactive fibers were seen in close proximity to VR1-immunoreactive neurons (Fig. 3G).

Myenteric plexus

The chemical coding of DOR (Fig. 4A–D) and VR1 (Fig. 4E–H)-immunoreactive neurons in the porcine ileal myenteric plexus (MP) was investigated. As in the submucosal plexuses, DOR and ChAT immunoreactivities were highly colocalized in myenteric neurons, although some DOR-immunoreactive neurons did not display ChAT immunoreactivity (Fig. 4A). Adjacent or nearby tissue sections coincubated with antibodies to NOS and ChAT revealed no colocalization of these neuronal markers (Fig. 4B). Although DOR- and VR1-immunoreactive

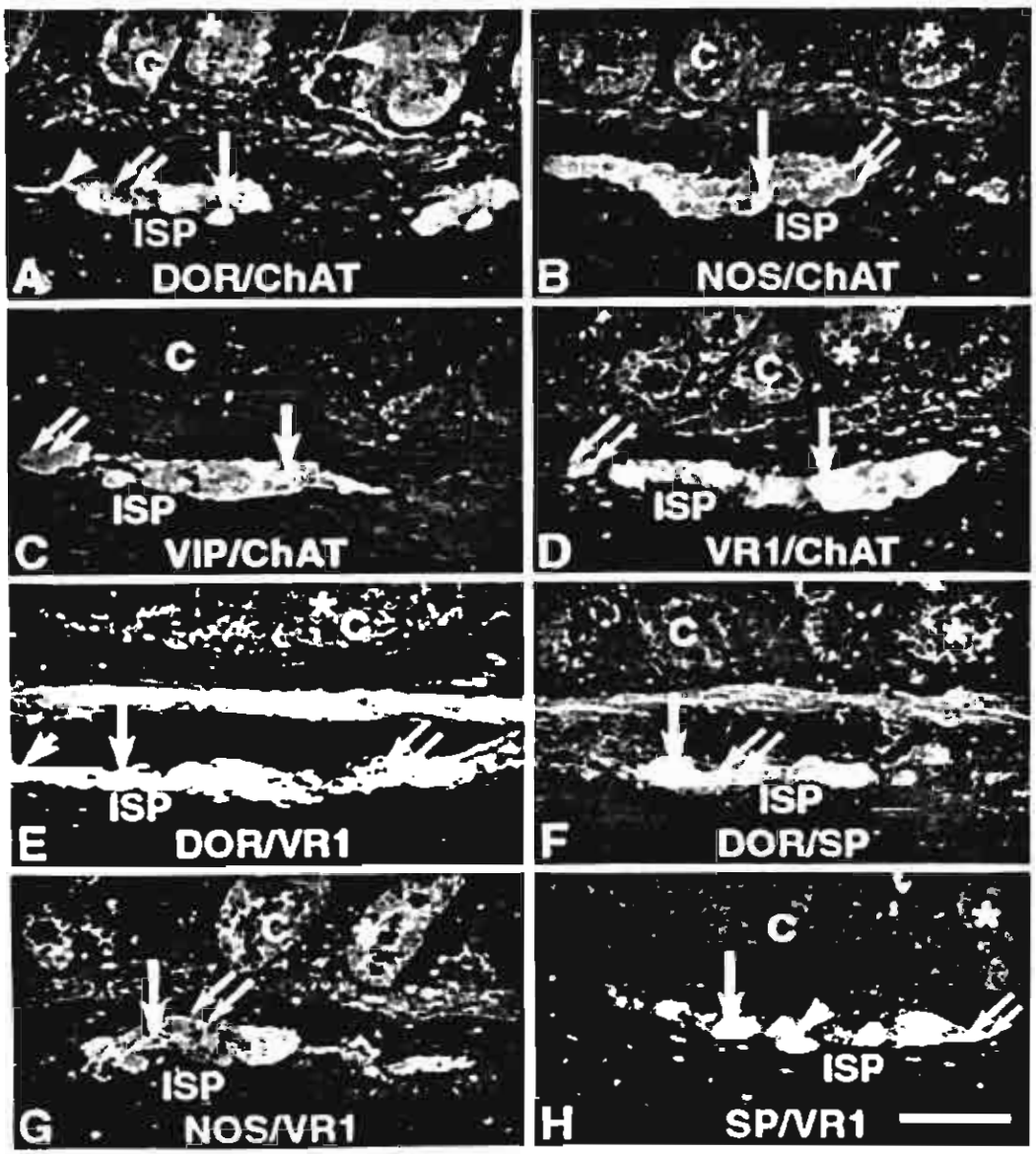

Fig. 2 Photomicrographs of representative longitudinal sections of porcine ileum demonstrating the chemical coding of δ-opioid receptor (*DOR*)-immunoreactive neurons (**A–C, F**) and type I vanilloid receptor (*VR1*)-immunoreactive neurons (**D, E, G, H**) in the inner submucosal plexus (*ISP*) of porcine ileum. **A** Colocalization (*yellow*) of DOR (*green*) and choline acetyltransferase (*ChAT, red*) immunoreactivities was observed in neurons (*vertical arrow*). Some of the DOR-immunoreactive nerve fibers in ISP (*arrowhead*) did not express ChAT immunoreactivity, and some neurons positive for ChAT (*double arrows*) did not express DOR immunoreactivity. **B–D** Because antineuronal nitric oxide synthase (*NOS*) and antivasoactive intestinal peptide (*VIP*) antisera were made in the same host, ChAT/DOR-immunoreactive neurons were compared with adjacent or nearby sections incubated with antibodies to ChAT and NOS (**B**) or VIP (**C**). No colocalization of ChAT immunoreactivity (*red*) with that of NOS (*green*, **B**) or VIP (*green*, **C**) was observed in neurons. **D** Adjacent section concubated with VR1 (*green*) and ChAT (*red*) antisera revealed colocalization in some neurons (*yellow*; *vertical arrow*), although some ChAT-positive neurons (*double arrows, red*) did not express VR1 immunoreactivity. **E** DOR-positive neurons (*green*) coexpressing VR1 immunoreactivity (*red*) were observed (*yellow*; *vertical arrow*). In addition, some DOR (*arrowhead*) and VR1 (*double arrow*)-immunoreactive neurons that did not exhibit colocalization were observed. **F** Section coincubated with anti-DOR (*green*) and anti-substance P (*SP, red*) antisera revealed that some DOR-immunoreactive neurons (*double arrow*) coexpressed SP immunoreactivity (*vertical arrow*; *yellow*). **G, H** Localization of VR1 (*red*, **G, H**) and NOS (*green*, **G**) or SP (*green*, **H**) immunoreactivities in ISP neurons. **G** NOS-positive neurons (*vertical arrow*; *green*) did not coexpress VR1 immunoreactivity (*double arrows, red*) and vice versa. **H** Substance P immunoreactivity (*green*) was occasionally colocalized (*vertical arrow*; *yellow*) with VR1 immunoreactivity (*double arrow*; *red*) in some ISP neurons. *Asterisks* denote nonspecific staining seen on or under the epithelium and muscularis mucosae in the section that persisted in omission and preabsorption control experiments (*C* crypt). *Scale bar* 200 μm

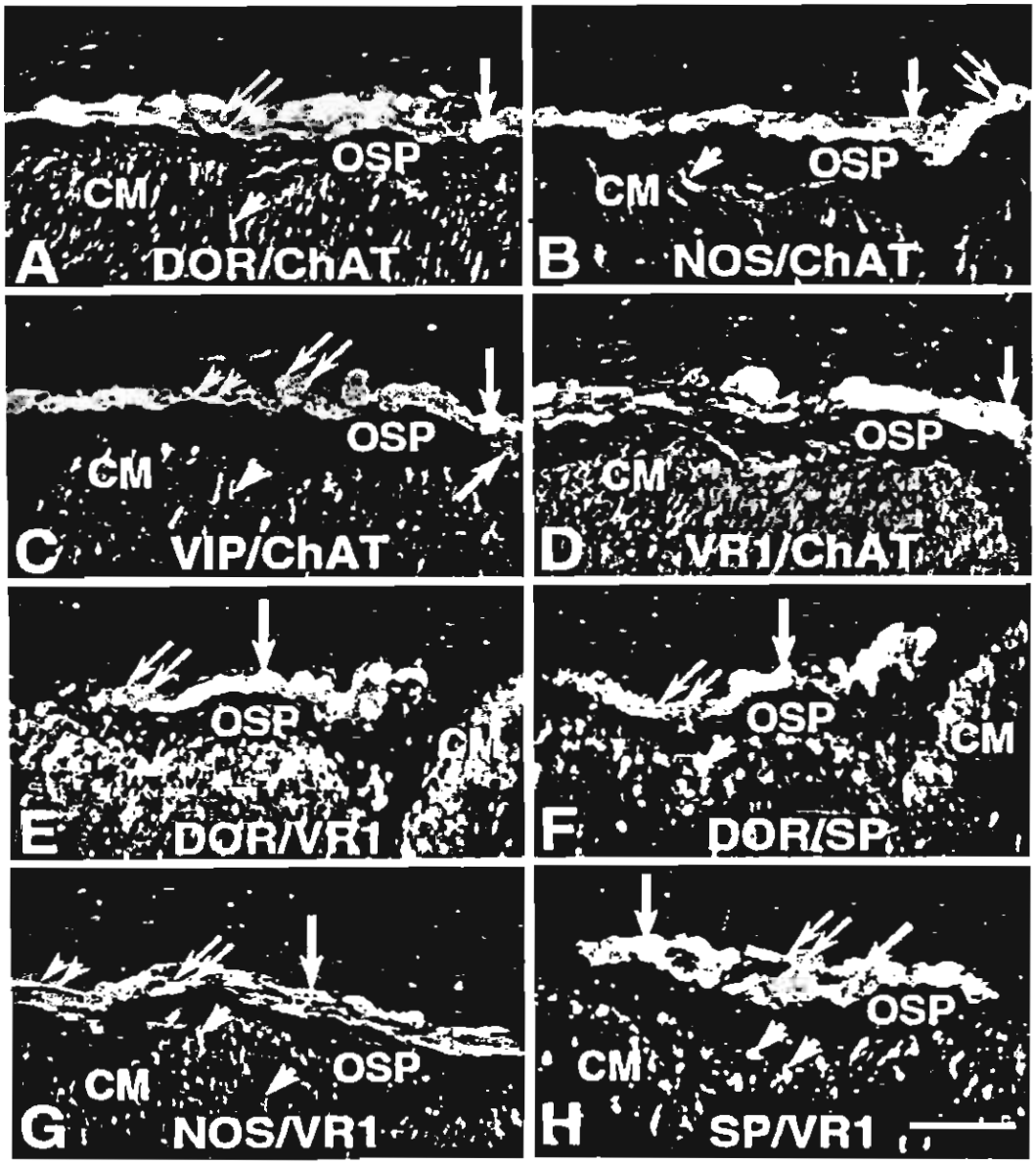

Fig. 3 Photomicrographs of representative longitudinal sections of porcine ileum showing the chemical coding of δ-opioid receptor (*DOR*)-immunoreactive neurons (**A–C**) and VR1-immunoreactive neurons (**D–H**) in outer submucosal plexus (*OSP*) of the porcine ileum. **A** Colocalization (*yellow*) of DOR (*green*) and choline acetyltransferase (*red*) immunoreactivity was seen in OSP neurons (*slanted arrow*) and also seen in nerve fibers in deep circular smooth muscle (*arrowhead, yellow*). Some ChAT-positive neurons did not co-contain DOR immunoreactivity (*double arrow, red*). **B, C** As explained earlier in Fig. 2, we compared DOR–ChAT-positive neurons in adjacent sections coincubated with anti-ChAT antiserum and anti-nitric oxide synthase (*NOS, green*, **B**) or anti-vasoactive intestinal peptide (*VIP, green*, **C**) antisera. **B** Colocalization of NOS (*green*) and ChAT (*red*) immunoreactivities was observed in a small number of neurons (*double arrow, yellow*) or nerve fibers (*arrowhead*), respectively. ChAT-immunoreactive neurons (*red*) as shown in **A** did not express NOS immunoreactivity (*vertical arrow, red*). **C** Most ChAT-immunoreactive neurons (*double arrow, red*) in the OSP are distinct from VIP-immunoreactive neurons (*slanted arrow, green*). A small population of ChAT-positive neurons coexpressed VIP immunoreactivity (*vertical arrow, yellow*). A few VIP-immunoreactive nerve fibers alone were seen (*arrowhead, green*). **D** Adjacent section costained with VR1 antiserum (*green*) and ChAT antiserum (*red*) revealed colocalization of VR1 and ChAT immunoreactivities (*vertical arrow, yellow*) in the same ChAT-positive neurons seen in **C** and expressed both VR1 and VIP immunoreactivities. **E, F** Double-staining of adjacent sections with anti-DOR (*green*, **E, F**), anti-VR1 (*red*, **E**) or anti-SP antiserum (*red*, **F**). **E** Colocalization of DOR and VR1 immunoreactivities was observed in some OSP neurons (*vertical arrow, yellow*). A few VR1-immunoreactive neurons (*double arrow, red*) and nerve fibers in circular smooth muscle (*arrowhead*) did not colocalize with DOR immunoreactivity. **F** In most neurons, DOR immunoreactivity (*green*) did not colocalize with SP immunoreactivity (*double arrows, red*). However, a few neurons may coexpress DOR and SP immunoreactivity (*vertical arrow, imparting some yellowish fluorescence*). Colocalization of DOR and SP immunoreactivities was seen in nerve fibers in smooth muscle (*arrowhead, yellow*). **G, H** Sections costained with VR1 (*red*, **G, H**) and NOS (*green*, **G**) or SP (*green*, **H**) antiserum. **G** Distinct localization of VR1 and NOS immunoreactivities was observed in OSP neurons (*vertical arrow and double arrow, respectively*), and nerve fibers in circular smooth muscle (*arrowhead*). **H** Colocalization of SP and VR1 immunoreactivities was seen in some OSP neurons (*vertical arrow, yellow*), but not in nerve fibers within circular muscle (*arrowhead*) (*CM* circular muscle). *Scale bar* 200 μm

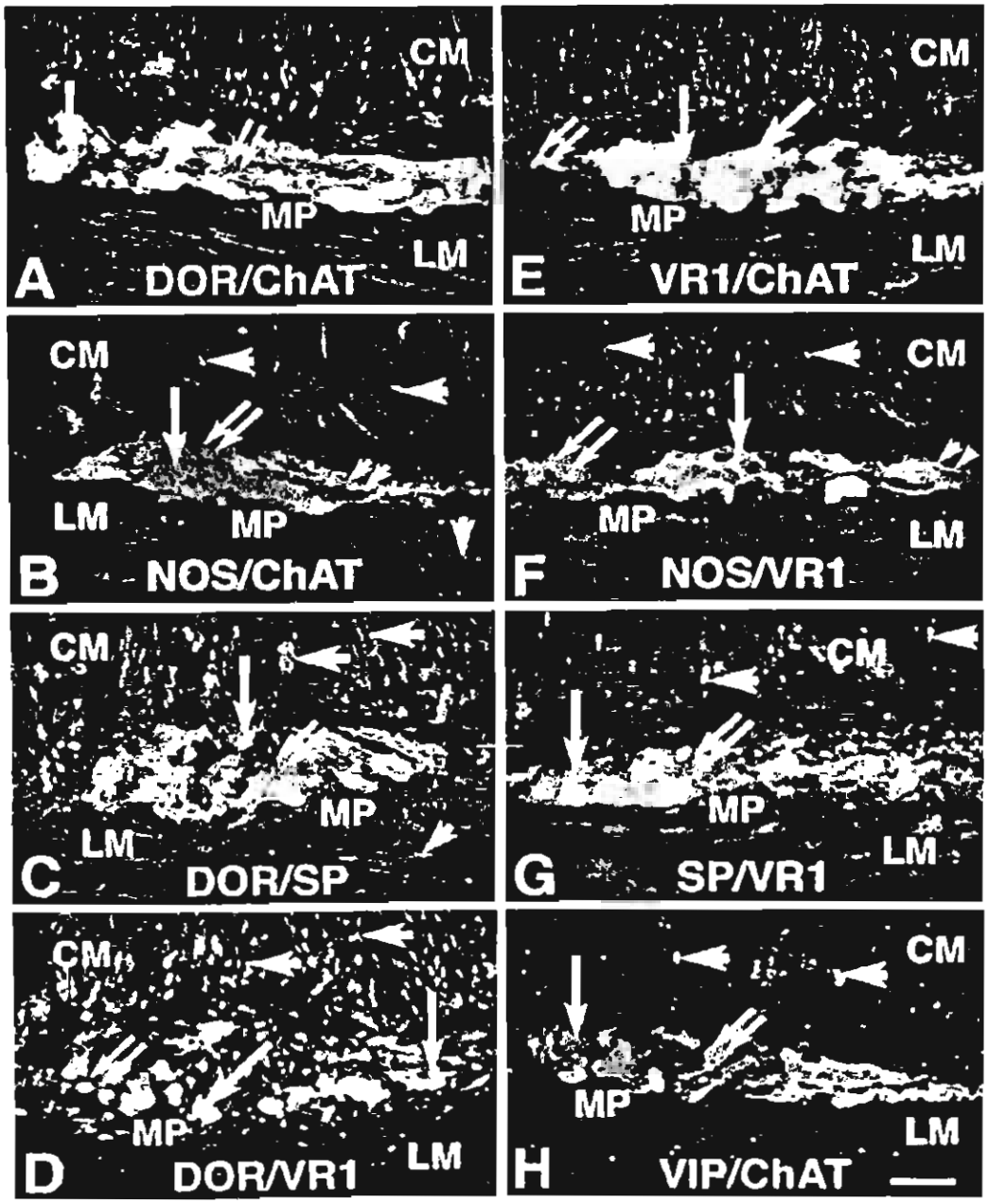

Fig. 4 Photomicrographs of representative longitudinal sections of porcine ileum depicting the chemical coding of δ-opioid receptor (*DOR*)-immunoreactive neurons (*green*, A–D, H) and VR1-immunoreactive neurons (*green* in E, *red* in F, G) in myenteric plexus (*MP*) of porcine ileum. **A** Colocalization (*slanted arrow, yellow*) of DOR (*green*) and choline acetyltransferase (*ChAT, red*) immunoreactivities was observed in neurons. Distinct populations of DOR-positive neurons alone (*vertical arrow, green*) and ChAT-positive neurons alone (*double arrow, red*) also were observed. **B, H** Colocalization of DOR and ChAT immunoreactivities was compared with those in adjacent or nearby sections concubated with ChAT (*red*, **B**) and neuronal nitric oxide synthase (*NOS, green*, **B**) antisera or ChAT (*red*, **H**) and vasoactive intestinal peptide (*VIP, green*, **H**) antisera. No colocalization of ChAT (*double arrow*) and NOS (**B**) or VIP (**H**) immunoreactivities was observed in neurons (*vertical arrow, green*, **B** or **H**). Nerve fibers in circular (*CM, arrowhead*) or longitudinal (*LM, arrowhead*) muscle expressed ChAT immunoreactivity. **C** Sections costained with DOR (*vertical arrow, green*) and substance P (*SP, double arrow, red*) antiserum revealed no colocalization in neurons or nerve fibers (*arrowheads*). **D** VR1 immunoreactivity in many neurons (*red*) was colocalized (*slanted arrow, yellow*) with DOR (*green*) immunoreactivity. Only a subpopulation of neurons (*vertical arrow, green*) or some nerve fibers (*arrowhead, green*) immunoreactive to DOR did not express immunoreactivity to VR1. Immunoreactivities of VR1 (*green*) with ChAT (*red*, **E**), VR1 (*red*) with NOS (*green*, **F**) and VR1 (*red*) with substance P (*SP, green*, **G**) in myenteric neurons and nerve fibers. **E** Many ChAT-positive neurons expressed VR1 immunoreactivity (*slanted arrow, yellow*), although some VR1-positive neurons (*vertical arrow, green*) did not express ChAT immunoreactivity (*double arrow, red*). **F, G** VR1-immunoreactive neurons and circular muscle nerve fibers (*double arrow and arrowhead, respectively, red*, **F, G**) not immunoreactive to NOS (*vertical arrow and arrowhead, green*, **F**) or SP (*vertical arrow and arrowhead, green*, **G**). NOS-immunoreactive fibers in the myenteric plexus (*double arrowheads, green*, **F**) also did not express VR1 immunoreactivity. *Scale bar* 150 μm

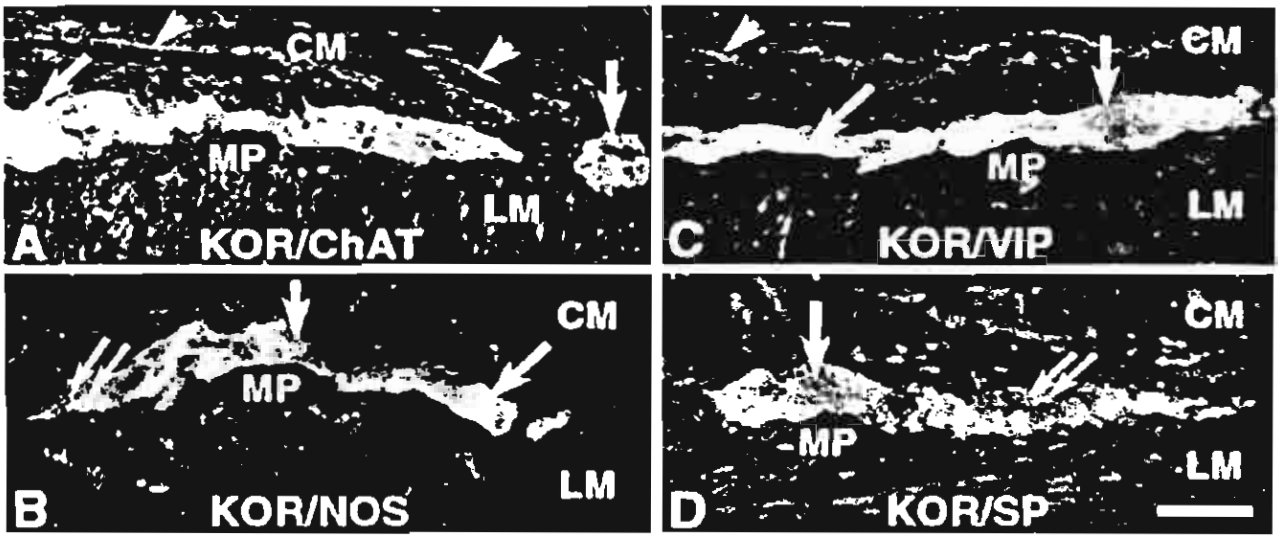

Fig. 5A–D Chemical coding of κ-opioid receptor (*KOR*)-immunoreactive neurons (*green*) in the myenteric plexus (*MP*) of porcine ileum. **A–C** are photomicrographs of representative transverse sections and **D** is a longitudinal section. **A** Colocalization (*slanted arrow, yellow*) of KOR (*green*) and choline acetyltransferase (*ChAT, red*) immunoreactivities was observed in neurons. Some ChAT-positive neurons (*vertical arrow*) did not express KOR immunoreactivity. Likewise, KOR-immunoreactive fibers in the circular (*CM*) and longitudinal (*LM*) smooth muscle did not express ChAT immunoreactivity (*arrowhead*). **B** Colocalization of KOR (*green*) and neuronal nitric oxide synthase (*NOS, red*) in neurons (*slanted arrow, yellow*). Although KOR and NOS immunoreactivities were colocalized in some neurons, most neurons were immunoreactive to KOR alone (*double arrow*) or NOS alone (*vertical arrow*). **C** Colocalization of KOR (*green*) with vasoactive intestinal peptide (*VIP, red*) immunoreactivities in myenteric neurons (*yellow, slanted arrow*). Some VIP-immunoreactive neurons (*vertical arrow*) did not exhibit KOR immunoreactivity. No colocalization was observed in nerve fibers (*arrowhead*). **D** Sections coincubated with KOR (*green*), and substance P (*SP, red*) antisera exhibited two distinct populations of neurons (*vertical arrow*) and nerve fibers in smooth muscle. *Scale bar* 100 μm

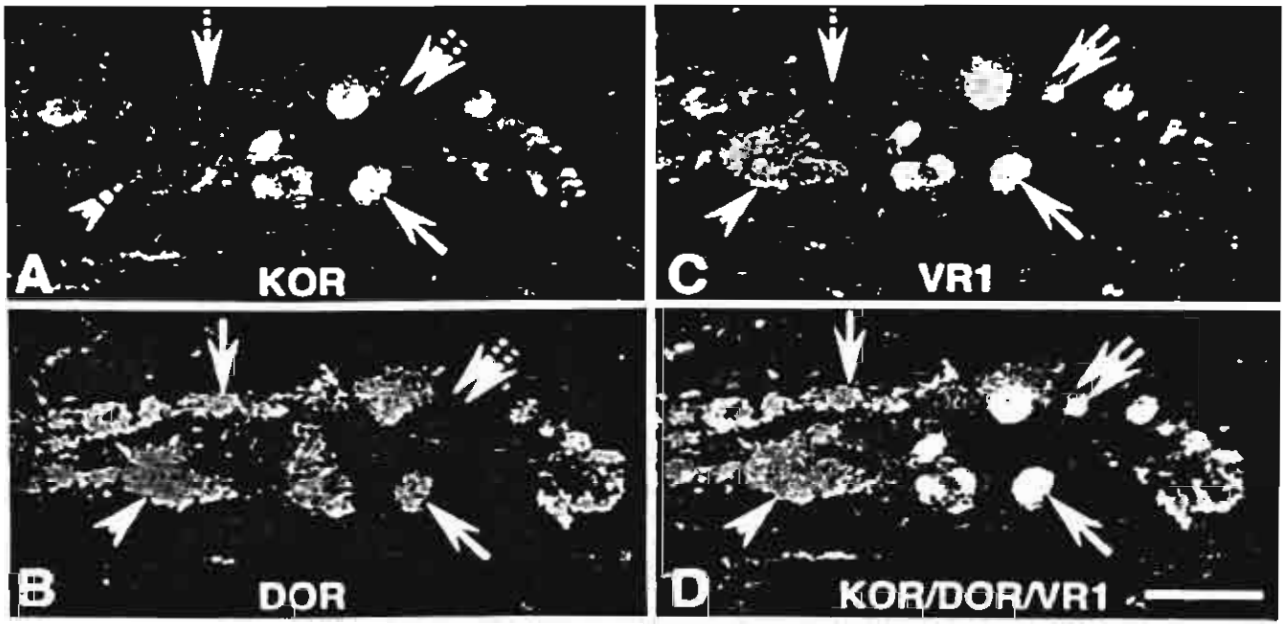

Fig. 6A–D Photomicrographs of representative longitudinal sections of porcine ileum showing colocalization of δ (*DOR*) and κ (*KOR*)-opioid receptor immunoreactivities with type I vanilloid receptor (*VR1*) immunoreactivity in porcine myenteric neurons. **A** KOR immunoreactivity was observed in small neurons (*slanted arrow, green*). **B** Localization of DOR immunoreactivity in the same neuron (*slanted arrow, blue*) as shown in **A**. Some neurons expressing DOR immunoreactivity (*arrowhead, blue*) did not express KOR immunoreactivity as shown in **A** (*arrowhead*). **C** Type I vanilloid receptor (*VR1*) immunoreactivity was observed in the same neuron as shown in **A** and **B** (*slanted arrow, red*). VR1 immunoreactivity also was observed in KOR-negative, DOR-positive neurons (*arrowhead*) as shown in **A** and **B**, respectively. **D** Photomicrograph showing colocalization of KOR, DOR, and VR1 immunoreactivities (*slanted arrow, off-white fluorescence*) in some neurons. DOR and VR1 immunoreactivities were coexpressed (*arrowhead, violet fluorescence*) in some KOR-negative neurons. *Broken arrow* denotes DOR-immunoreactive neuron that does not express KOR (**A**) or VR1 (**C**) immunoreactivity. Some VR1-immunoreactive neurons (*double arrow*) did not express immunoreactivity to opioid receptors (**A** and **C**, *broken double arrow*). *Scale bar* 50 μm

neurons did not coexpress immunoreactivity to SP (Fig. 4C and G, respectively), immunoreactivities to these two receptors were highly colocalized in MP neurons (Fig. 4D, Table 2). VR1 immunoreactivity was highly colocalized with ChAT immunoreactivity in MP neurons (Fig. 4E, Table 2), but not with NOS and SP immunoreactivities (Fig. 4F and G, respectively). ChAT-positive neurons did not appear to coexpress VIP immunoreactivity (Fig. 4H).

KOR immunoreactivity was colocalized in nearly half of ChAT-positive myenteric neurons examined (Fig. 5A, Table 2). Unlike the distribution patterns of DOR or VR1 immunoreactivities in MP, some KOR-positive neurons expressed NOS or VIP immunoreactivities (Fig. 5B and C, respectively, Table 2). There was no colocalization between KOR and SP immunoreactivity in MP neurons (Fig. 5D). Immunoreactivities to KOR (Fig. 6A), DOR (Fig. 6B) and VR1 (Fig. 6C) were colocalized (Fig. 6D) in some myenteric neurons.

Discussion

As previously reported, DOR immunoreactivity was localized in neurons and fibers of all enteric plexuses within the wall of the porcine ileum (Brown et al. 1998). In contrast, immunoreactivity to KOR was confined to the myenteric plexus. A predominant localization of KOR immunoreactivity in MP neurons and nerve fibers has also been reported in the rat small intestine and colon (Bagnol et al. 1997). Immunoreactivity to DOR appeared to be expressed predominately in ChAT-positive enteric neurons. KOR immunoreactivity, on the other hand, appeared to be expressed by ChAT-positive neurons as well as VIP- or NOS-immunoreactive neurons in myenteric ganglia. Both DOR and KOR in the MP may modulate intestinal motor function. Functional studies in a circular muscle-MP preparation from porcine ileum have shown that stimulation of DOR or KOR with receptor-selective agonists is associated with an inhibition of neurogenic contractions (Brown et al. 1998; Poonyachoti et al. 2001a). In agreement with the presence of DOR immunoreactivity, but not KOR in the submucosal neurons, it has recently been reported that DOR-selective agonists potently suppress neurogenic anion transport in porcine ileal mucosa containing an intact ISP; KOR-selective agonists were nearly 1000-fold less potent in this preparation (Poonyachoti et al. 2001b). There is no functional or immunohistochemical evidence for the expression of μ-ORs in porcine ileum (Poonyachoti et al. 2001a, 2001b). This stands in contrast to the rat and guinea pig small intestine in which μ-OR immunoreactivity was observed to predominate in enteric neurons and nerve fibers (Bagnol et al. 1997; McConalogue et al. 1999), and probably represents a major species difference in OR expression. Immunoreactivity to one class of endogenous OR ligands, the enkephalins, is prominent in neurons and fibers of the porcine MP, but is relatively sparse in the submucosal plexuses (Porcher et al. 2000). These opioid peptides are therefore likely to activate myenteric ORs under physiological conditions. It is possible that another class of endogenous ligands interacts with DOR expressed in the ISP, a receptor with pharmacological characteristics that are quite different from DOR in the MP (Poonyachoti et al. 2001b).

VR1 immunoreactivity, like that to DOR, was distributed in all three ganglionated plexuses of porcine ileum, where it was also highly colocalized in ChAT-immunoreactive neurons. Its expression in the perikarya and fibers of enteric neurons is similar to its immunocytochemical pattern of expression in dorsal root ganglion neurons (Guo et al. 1999). There was a significant colocalization of DOR and VR1 immunoreactivities in neurons within all three enteric plexuses as well as triple colocalization of KOR, DOR and VR1 immunoreactivities in some KOR-positive myenteric neurons examined. The colocalization of KOR and DOR immunoreactivities on these neurons may be indicative of a functional association between these receptors. Indeed, Jordan and Devi (1999) have proposed the existence of functional KOR/DOR heterodimers based on studies of recombinantly expressed receptors in cultured cells. There are clearly other distinct populations of MP neurons that express DOR, KOR or VR1 immunoreactivity alone or in combination. We have recently confirmed the colocalization of DOR and KOR as well as DOR and VR1 immunoreactivities in primary cultures of porcine myenteric neurons (Kulkarni-Narla and Brown 2001). The functional interactions of these receptors on enteric neurons remain to be explored.

Immunoreactivity to DOR or VR1 was frequently colocalized in enteric neurons displaying immunoreactivity to the sensory neuropeptide CGRP. In addition, KOR and CGRP immunoreactivities were highly coexpressed in myenteric neurons. Immunoreactivity to DOR has been colocalized with CGRP immunoreactivity in dorsal root ganglion neurons and fibers (Dado et al. 1993; Guo et al. 1999). Moreover, KOR- and DOR-positive dorsal root ganglion neurons are sensitive to the VR1 agonist capsaicin (Zhang et al. 1998) and activation of KOR decreases CGRP release into the intrathecal space (Collin et al. 1993). VR1 and CGRP immunoreactivities are highly colocalized in trigeminal ganglia (Ichikawa and Sugimoto 2001), and the VR1 agonists capsaicin and anandamide evoke CGRP release from nerve terminals in the dorsal spinal cord (Tognetto et al. 2001). In vascularly perfused, isolated segments of porcine ileum, capsaicin infusion is associated with a marked increase in CGRP release (Rasmussen et al. 2001). We hypothesize that ChAT-/CGRP-positive enteric neurons that coexpress OR and VR1 immunoreactivities may represent intrinsic primary afferent neurons (IPANs) which coordinate enteric secretomotor and vasodilatory reflexes (Furness et al. 1998). In rat and guinea pig small intestine, IPANs manifest ChAT immunoreactivity (Li and Furness 1998; Mann et al. 1999) as do CGRP-immunoreactive Dogiel type 2 neurons in the porcine small intestine (Scheuermann et al. 1987; Hens et al. 2000). Immu-

noreactivity to another putative sensory transmitter, SP, was not detected in DOR-, KOR- or VR1-positive MP neurons although small populations of SP-positive submucosal neurons were immunoreactive for either DOR or VR1, these latter neurons may have a secretomotor role in the porcine intestine (Hens et al. 2000). The functional roles served by VR1-immunoreactive neurons in the enteric nervous system await additional investigations

In the porcine small intestine, both the OSP and MP have considerably fewer mucosal projections than the ISP (Hens et al. 2000). Neurons in these two plexuses innervate the intestinal smooth muscle layers and send viscerofugal projections to the prevertebral ganglia (Timmermans et al. 1997). It has been suggested that the OSP and MP of the porcine small intestine possess similar morphological features and neurochemical coding that differ from the ISP (Timmermans et al. 1997; Hens et al. 2000). In the present study, there was substantial overlap between VR1 and SP immunoreactivities in OSP neurons, but little or no colocalization of these two immunoreactivities in MP neurons. The OSP and MP appeared to differ from each other in the expression of KOR immunoreactivity, which was only detected in MP neurons expressing immunoreactivity to either ChAT, CGRP, NOS or VIP. These results indicate that there are some differences in the receptor signatures of chemically coded neurons in these two ganglionated plexuses

Immunoreactivity to KOR, but not to DOR and VR1, was expressed on VIP- or NOS-immunoreactive myenteric neurons. NOS-immunoreactive neurons have previously been reported to predominate in the OSP and MP of porcine small intestine (Timmermans et al. 1994). Their presence in the ISP neurons observed in the present study may reflect segmental differences in NOS expression along the length of the intestine. The presence of KOR immunoreactivity on myenteric neurons containing inhibitory neurotransmitters may account for functional differences in the effects of selective δ- and κ-OR agonists on intestinal motility. Selective κ-OR agonists inhibit atropine-sensitive "on" contractions evoked by electrical field stimulation in circular muscle-MP strips from porcine ileum, whereas δ-OR agonists predominately reduce atropine-sensitive "off" contractions in this preparation that occur immediately after the cessation of electrical stimulation (Poonyachoti et al. 2001a). Neurons coexpressing opioid and vanilloid receptors that are situated in the OSP and MP may modulate intestinal segmentation and propulsion. Moreover, DOR, KOR and VR1 immunoreactivities also could be expressed by IPANs in the OSP and MP that sense luminal chemicals, mucosal distortion or intestinal distension and evoke mechanical reflexes to assist the digestive process or speed the elimination of luminal pathogens.

Nearly 80% of neurons with axonal projections to the intestinal mucosa reside in the ISP of porcine ileum, about half of these include distinct subpopulations of ChAT-immunoreactive neurons (Hens et al. 2000). DOR and VR1 immunoreactivities may be localized on one or more subsets of ChAT-positive, VIP-negative ISP neurons with mucosal projections that have been reported in porcine small intestine; in addition to large type 2 sensory neurons with a ChAT/CGRP (SP) phenotype, these neurons include a major subpopulation of small ChAT/SP-immunoreactive neurons and a very small population of multidendritic ChAT/somatostatin-immunoreactive neurons which have putative secretomotor roles (Hens et al. 2000).

We hypothesize that DOR modulates a neural pathway in the porcine ileal submucosa that links intestinal inflammation and hypersensitivity with transepithelial secretion, an important mucosal defense mechanism (Perdue and MacKay 1994). DOR and VR1 immunoreactivities are expressed in CGRP-positive ISP neurons, and there is increasing evidence that imbalances in the enteric neural circuits containing sensory transmitters such as CGRP are involved in the pathophysiological alterations of mucosal secretion, blood flow and immune function that are associated with intestinal infection, allergy and inflammation (Holzer 1998). Neurons in the ISP coexpressing CGRP, VR1 and DOR may play an important role in sensing and modulating these pathophysiological changes in mucosal function. In the guinea pig ileum for example, the VR1 agonist capsaicin induces active anion secretion and increases submucosal blood flow (Vanner and MacNaughton 1995; Vanner and Bolton 1996). Enteric neurons expressing opioid and vanilloid receptors may constitute important therapeutic targets for the development of drugs alleviating painful intestinal inflammatory, allergic and dysmotility states. Drugs interacting with DOR or VR1 may be useful in alleviating some of the manifestations of inflammatory bowel disease. Abdominal pain is associated with inflammation, anaphylaxis or ischemia and submucosal nerves that express DOR may mediate the analgesic actions of opioids in these conditions (Bueno et al. 2000). Finally, the roles of DOR and VR1 submucosal neurons in modulating mast cell and immunocyte function as well as overall disease resistance in the intestinal mucosa certainly deserve examination (Downing and Miyan 2000).

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