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Opioid, cannabinoid and vanilloid receptor localization on porcine cultured myenteric neurons

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Abstract

Opioids and cannabinoids have profound inhibitory actions on intestinal motility which are mediated in part by their cognate receptors in the enteric nervous system. In the present study, we examined the expression of immunoreactivity for δ- and κ-opioid receptors, CB_1-cannabinoid receptors and type 1 vanilloid receptors by immunocytochemistry and confocal laser scanning microscopy on ileal myenteric neurons, isolated from juvenile pigs, that were < 70 μm diameter in either axis and maintained for 1–2 weeks in primary culture. Immunoreactivities for δ-opioid and cannabinoid receptors were present in neurons immunoreactive for the cholinergic marker, choline acetyltransferase. Some neurons with δ-opioid receptor-like immunoreactivity were also immunoreactive for κ-opioid, cannabinoid or vanilloid receptors. These observations indicate that receptors for cannabinoids or vanilloids are co-localized in opioid receptor-expressing myenteric neurons which modulate intestinal sensorimotor function.

Keywords: Gut; Enteric nervous system; Myenteric plexus; Receptors; Pig

The enteric nervous system regulates patterns of motility in the intestine, including the regional mixing and propulsion of gut contents, and propagated contractions associated with the migrating myoelectric complex [12]. The ability of opioids, such as codeine and loperamide, to inhibit intestinal propulsion and slow intestinal transit is a prominent component of the antidiarrheal and constipating actions of this drug class. Presynaptic opioid receptors (ORs) on intrinsic neurons in the myenteric plexus mediate opioid-induced neuronal hyperpolarization and reductions in the release of acetylcholine and other neurotransmitters through the G protein-coupled activation of K^+ channels or inhibition of N-type Ca^{2+} channel gating [7]. Like the opioids, botanical, endogenous and synthetic cannabinoids have been shown to inhibit neurotransmitter release and decrease intestinal motility through interactions with presynaptic CB_1-cannabinoid receptors (CB_1-R) in the myenteric plexus [4,5,10]. In the porcine small intestine, a biomedical model for the human intestine, opioid agonists acting selectively at δ-ORs (DOR) or κ-ORs (KOR) decrease neurogenic contractions of smooth muscle-myenteric plexus strips from porcine ileum [13]. In addition to these ORs, the porcine myenteric plexus exhibits relatively high levels of expression for their cognate ligands, the enkephalins [14]. In support of these data, DOR and KOR immunoreactivities are expressed in myenteric neurons and fibers examined in tissue sections; in some neurons, these receptors appear to be colocalized [3,13]. In addition, CB_1-R immunoreactivity has been found to be expressed in neurons within myenteric ganglia of the porcine ileum and colon that are also immunoreactive for the acetylcholine synthesizing enzyme, choline acetyltransferase (ChAT) [11].

The availability of myenteric neurons in primary culture would enhance investigations of the biology and potential interactions between enteric opioid and cannabinoid receptors at the cellular and molecular level. In the present study, we developed a method for maintaining myenteric neurons from juvenile pigs in primary culture to test the hypothesis that under culture conditions, these neurons would manifest the opioid and cannabinoid receptor signatures observed in our previous immunohistochemical experiments in porcine small intestine [3,11,13]. As DOR immunoreactivity has been found in primary afferent fibers in dorsal horn of the spinal cord [6], it was of additional interest to examine the possible colocalization of these receptors on myenteric neurons with the type 1 vanilloid receptor (VR1), a

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ligand-gated ion channel which plays an important role in sensory neurotransmission [16].

Intestinal tissues were obtained from five Yorkshire pigs (6–10 weeks of age; 10–18 kg body weight) of each sex which were not fasted before sacrifice. Animals were sedated with an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol®; 8 mg/kg, Fort Dodge Laboratories, Fort Dodge, IA), in combination with xylazine (8 mg/kg). The animals were subsequently euthanized by barbiturate overdose in accordance with approved University of Minnesota Animal Care Committee protocols. The ileum was isolated and prepared as described previously [11]. The muscularis externa containing the myenteric plexus was separated from the mucosa-submucosa by blunt dissection and immersed in chilled phosphate-buffered saline (PBS, pH 7.4). All chemicals for cell culture were purchased from Life Technologies (Gaithersburg, MD) unless otherwise specified. The tissue was minced and incubated in 0.05% trypsin and 0.53 nM EDTA in Hank's balanced salt solution for 90 min at 37°C, under an atmosphere of 90% air/10% CO_2. Trypsin was inactivated by adding Dulbecco's modified Eagle's medium (DMEM; 4.5 mg/ml glucose, 1 nM sodium pyruvate, 1 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin) containing heat-inactivated fetal bovine serum (FBS). Digested tissue was then filtered through a metal sieve (40 μm mesh size; Fisher Scientific, Pittsburgh, PA) and discarded. Cells were pelleted by centrifugation at 1200 rpm for 15 min and resuspended in the same medium as described above. The cell suspension was filtered through a cell strainer (70 μm pore size; Becton Dickinson, Franklin Lakes, NJ) and neurons below 70 μm in size were collected. Cells were pelleted twice by centrifugation and resuspended in DMEM containing B27 supplement in place of FBS. Petri dishes (35 mm) coated with human laminin were seeded at 1.5×10^5 cells/well in 1 ml of DMEM/B27 containing 2.5 μg/ml fungizone. All procedures were done aseptically. Cultures were maintained at 37°C for 1–3 weeks, with the culture medium changed on alternate days. To promote neuronal growth, 40 ng/ml of neurotropin 3 (NT3; R & D Systems, Minneapolis, MN) was added to the culture medium of some cells; to prevent fibroblast growth, 100 mM cytosine arabinoside (Sigma Chemical Co., St. Louis, MO) was added to all cell cultures after 3 days in culture.

Cells maintained under culture conditions for 1–3 weeks were fixed in 2% paraformaldehyde for 10 min and washed with PBS and processed for immunocytochemistry. PGP 9.5 and ChAT immunoreactivities were detected with rabbit anti-human PGP 9.5 antiserum (1:1000 dilution; Neuromics, Minneapolis, MN) and goat anti-human ChAT antiserum (1:30, Chemicon International, Temucula, CA), respectively. DOR immunoreactivity was detected with either a rabbit polyclonal antiserum (1:1000 dilution) against an extracellular sequence (PFQSAKYLMETWPFGELL) of the mouse DOR that is conserved in porcine DOR and generously provided by Dr Robert P. Elde [1], or a commercial goat polyclonal antiserum (1:500 dilution) raised against the N-terminus of human DOR (DOR-1, Santa Cruz Biotechnology, Santa Cruz, CA). KOR immunoreactivity was visualized with a goat polyclonal antiserum (1:600 dilution) obtained from Santa Cruz Biotechnology that was raised against the N-terminus of the human KOR. A rabbit polyclonal antiserum (1:600 dilution) raised against an extracellular N-terminal sequence of the human CB_1-R (MKSILDGLADTTFR) was purchased from Cayman Chemicals (Ann Arbor, MI). Type 1 vanilloid receptor (VR1) immunoreactivity was detected using a polyclonal antiserum (1:400 dilution) raised in guinea pigs against the C-terminus of murine VR1 that was provided by Dr Robert P. Elde [8]. A standard immunofluorescence staining protocol was followed. Briefly, cells were incubated in 0.4% Triton X-100 (Sigma Chemical Co., St. Louis, MO) and 2% bovine serum albumin (BSA, Sigma) in PBS for 30 min to block non-specific binding. Cell cultures were simultaneously incubated with antibodies against PGP9.5/ChAT, CB_1-R /ChAT; DOR/ChAT, DOR/KOR, DOR/CB1-R and DOR/VR1 in 0.4% Triton X-100 and 2% BSA overnight at 4°C. After three rinses in PBS, cells were simultaneously incubated with appropriate secondary antibodies (donkey anti-rabbit, -guinea pig, or -goat Cy3-conjugated IgG at 1:400 dilution and donkey anti-rabbit or -goat FITC-conjugated IgG at 1:50 dilution) in PBS for 1 h in the dark. After three rinses in PBS for 15 min, coverslips were mounted with Vectashield™ (Vector Laboratory, Burlingame, CA). As previously described [3,11,13], control experiments included the omission of primary antibodies from the staining protocol or the preabsorption of primary antibodies with their relevant antigens which resulted in absence of immunoreactivity in neurons.

A minimum of three culture dishes from three different animals were analyzed for each condition. Immunoreactive cells were scanned using a BioRAD confocal laser-scanning microscope (CLSM; Model 1024) which was attached to a Nikon fluorescence microscope. 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).

No overt differences in the expression of receptor or choline acetyltransferase (ChAT) immunoreactivities were observed on the cells maintained in primary culture for 1–3 weeks. Moderate neurite outgrowth was observed in some cultures to which NT3 was added. In general, the pattern of immunoreactivities and colocalization of the different antigens examined in this study corresponded with those observed previously in intact smooth muscle-myenteric plexus preparations from the ilea of juvenile pigs [11,13].

Representative micrographs of cells maintained in culture for 2 weeks in the presence of NT3 are shown in Fig. 1. Cells exhibiting PGP 9.5 immunoreactivity exhibited a neuronal profile (Fig. 1A). Both multidendritic neurons and smaller, bipolar neurons exhibited CB_1-R immunoreactivity (Fig. 1B). DOR-positive cells resembling Dogiel type

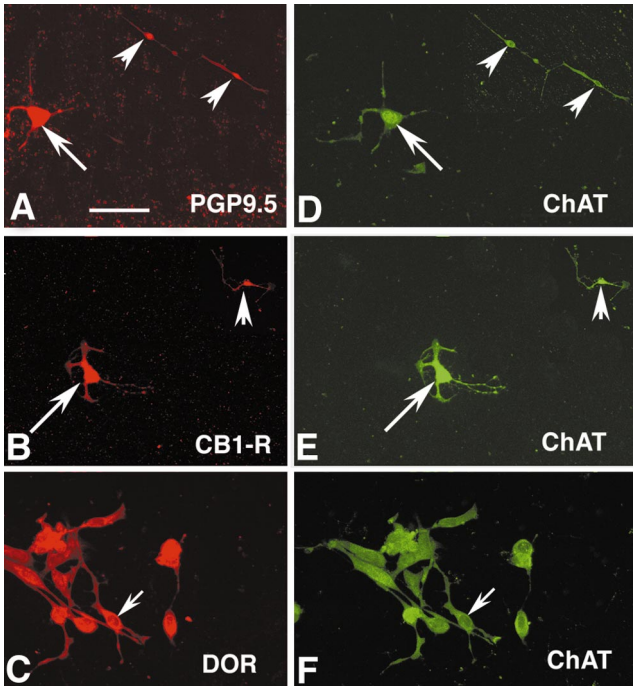

Fig. 1. (A-F) Chemical coding of porcine myenteric neurons maintained for 2 weeks in NT3-supplemented primary culture. (A) PGP 9.5 immunoreactivity is observed in large neurons with neuronal processes and mini-neurons (arrowheads). (B) A large pyramidal neuron with branched neurites (arrow) and mini-neuron (arrowhead) expressing CB_1-R immunoreactivity can be observed. (C) DOR immunoreactivity is observed in small, ovoid neurons with one or two processes (arrow). (D–F) ChAT immunoreactivity is observed in corresponding neurons expressing immunoreactivity to PGP 9.5 (A), CB_1-R (B) or DOR (C). Bar = 100 μm.

II neurons were ovoid in appearance with one or two processes (Fig. 1C). In the microscopic fields represented in Fig. 1, the size-selected neurons immunoreactive for the cholinergic marker, ChAT (Fig. 1D–F) were also immunoreactive for PGP 9.5 (Fig. 1A), CB_1-R (Fig. 1B) or DOR (Fig. 1C). In a previous study, ChAT-immunoreactive neurons in sections of porcine ileum also expressed CB_1-R immunoreactivity [11].

In cells cultured for 1 week in the absence of NT3, little neurite outgrowth could be observed. Nevertheless, these neurons manifested immunoreactivity to DOR (Fig. 2A–C). These relationships were also observed in myenteric neurons maintained in culture for 2 weeks. Subpopulations of these DOR-immunoreactive neurons co-expressed immunoreactivity to KOR (Fig. 2A), CB_1-R (Fig. 2B) or VR1 (Fig. 2C). KOR, CB_1-R and VR1 immunoreactivity was colocalized with DOR immunoreactivity in approximately 10, 30 and 55% of the total number of immunoreactive neurons, respectively in each microscopic field shown in Fig. 2.

We have recently reported that DOR and KOR immunoreactivities are colocalized in a small subset of porcine myenteric neurons in situ [13]. Although it is tempting to speculate that this pattern of receptor co-expression implies the existence of DOR-KOR heterodimers, this hypothesis that native heterodimeric ORs exist awaits biochemical and functional verification. Immunoreactivity for CB_1-R has previously been reported in porcine myenteric neurons in situ [11], but the co-localization of DOR and CB_1-R has not hitherto been described. Clearly, the phenotypic characteristics of myenteric neurons vis-à-vis these receptor populations are preserved in short-term culture.

There have been no previous reports of the expression of

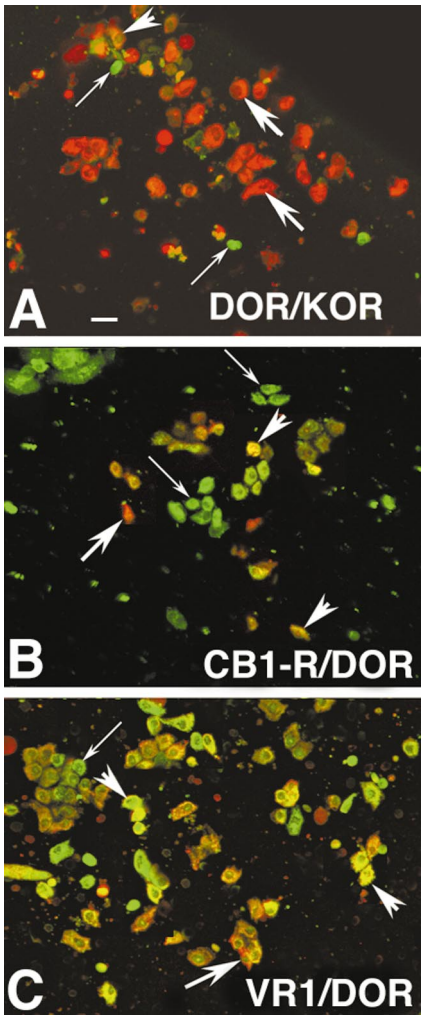

Fig. 2. (A-C) Chemical coding of porcine myenteric neurons maintained for 1 week in primary culture with no NT3 supplementation. (A) Colocalization (yellow, arrowhead) of DOR (red, large arrows) and KOR (green, small arrows) immunoreactivities is observed in myenteric neurons. Note that the number of neurons exhibiting DOR (red) immunoreactivity alone is greater than those displaying KOR immunoreactivity (green). (B) Colocalization (yellow, arrowheads) of CB_1-R (red, large arrow) and DOR (green, small arrow) immunoreactivities is observed in some neurons. Note the larger proportion of DOR-immunoreactive cells (green) immunoreactivity relative to the number of CB_1-R positive neurons (red). (C) Colocalization (yellow, arrowheads) of VR1 (red, large arrow) and DOR (green, small arrow) immunoreactivities can be seen in some myenteric neurons. Some neurons exhibited immunoreactivity only for DOR (green) or VR1 (red). Bar = 100 μm.

VR1 immunoreactivity on enteric neurons, but localized capsaicin infusions have been shown to stimulate motility in vascularly-perfused segments of porcine ileum [2,15]. The apparent neuronal colocalization of DOR and VR1 immunoreactivities suggests that DOR may modulate the activity of enteric sensory neurons sensitive to capsaicin and protons; these may include the small number of myenteric neurons with mucosal projections in the porcine small intestine [9]. Future investigations will explore the functional interrelationships of opioid, cannabinoid and vanilloid receptors in cultured myenteric neurons from the porcine ileum.

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