Distribution of vasoactive intestinal peptide and substance P messenger ribonucleic acid in intestinal nerve of Remak of chicken[1]

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ABSTRACT The intestinal nerve of Remak (INR) is unique to birds. The exact distributions and secretions of the peptide transmitters in the INR that innervate the intestine, and the patterns of their projections to the nerve plexus of the intestinal wall, are still unclear. In the present study, the distributions of chicken vasoactive intestinal peptide (VIP) mRNA and substance P (SP) mRNA in the chicken INR were demonstrated by means of in situ hybridization histochemistry, using sense and antisense digoxigenin-labeled RNA probes. The in situ hybridization histochemistry results suggested that VIP mRNA-positive neurons were mainly distributed in ganglions, whereas some of the INR fibers were weakly positive. The proportion of VIP mRNA-positive cells among total neurons was 75% in the juxta-jejunoileum portion and 87% in the juxta-rectal portion of the INR. Most SP mRNA-positive cells were sporadically distributed in layers or small groups within the ganglions, as well as in nerve trunks between ganglions. Moreover, the proportion of positive cells among total neurons was 83% in the juxta-jejunoileum portion and 98% in the juxta-rectal portion of the INR, which suggests that both VIP and SP mRNA are coexpressed in the chicken INR. Our results provide an approach to further study of the locations of VIP and SP in nerve tissue.

Key words: chicken intestinal nerve of Remak, vasoactive intestinal peptide messenger ribonucleic acid, substance P messenger ribonucleic acid, in situ hybridization, ribonucleic acid probe

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INTRODUCTION

The enteric nerve system (**ENS**), the third part of the autonomic nerve system, is independent of the central nervous system, in that it appears to be an entirely reflex pathway within the ENS. The avian ENS is composed of the intramural plexuses and intestinal nerves of Remak (**INR**). Because of the existence of this unique nerve, the INR, the route by which extrinsic nerves reach the intestine, is different in birds from that in other animals. The INR is principally derived from sacral neural crest cells that migrate throughout the gastrointestinal tract before differentiating into neurons and glia (Burns and Douarin, 1998). Commencing at the jejunal-duodenal border, it traverses the mesentery and mesorectum and runs parallel to the gut until it terminates at the cloaca. It sends many nerve branches to the intestine (Hodgkiss, 1984; Lunam, 1993; Suzuki et al., 1996). The INR innervates not only the intestine but also the ovaries (Dong et al., 1991), the bursa of Fabricius (Cui and Xie, 1990), the didymus (Wang, 1992), the magnum and shell gland of the oviduct (Chen et al., 2003; Liu et al., 2007b), and the cloaca (Chen et al., 1990). Previous studies demonstrated that the INR of the chicken was composed of sympathetic and parasympathetic nerves (Liu et al., 2007a). There are also nonadrenergic, noncholinergic neurons in the INR (Bartlett and Hassan, 1971; Ishizuka et al., 1982; Komori and Ohashi, 1982, 1987; Komori et al., 1988). The exact distributions and secretions of peptide transmitters in the intestinal wall that are innervated by INR nerve fibers, and the patterns of INR projection to the nerve plexus of the intestinal wall in the chicken, are still unknown.

Vasoactive intestinal peptide (**VIP**), containing 28 amino acids, is a kind of neuropeptide that is widely distributed in the avian digestive and genital tracts, and the bursa of Fabricius (Epstein et al., 1983; Epstein and Poulsen, 1991; Costagliola et al., 1997). In the digestive tract, the VIP mainly serves as a neurotransmitter in the intestinal wall neurons (Sundler et al., 1979). Substance P (**SP**) is a member of the tachykinin family, which comprises 11 amino acids; it is distributed mainly in the nervous system and other peripheral organs and tissues, but an appreciable amount

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is distributed in the intestinal wall (Lu, 2000). As a noncholinergic neurotransmitter, SP plays an important role in maintaining the normal physiological functions of the gastrointestinal tract. It has an excitatory effect on intestinal smooth muscle and influences the secretion of digestive juices.

Many immunohistochemical, electrophysiological, and pharmacological studies of the distribution of VIP and SP in the INR of chickens have been undertaken but have yielded many contradictory results (Epstein and Poulsen, 1991; Lunam, 1991; Suzuki et al., 1994; Aisa et al., 1997, 1998). Our studies are meant to provide some morphological evidence at the gene level that could elucidate the regulatory mechanisms of digestion, immunization, and reproduction mediated by the INR. Sense and antisense digoxigenin (**DIG**)-labeled RNA probes were designed to detect the distributions of VIP and SP mRNA in the INR, by means of in situ hybridization histochemistry (**ISHH**).

MATERIALS AND METHODS

All protocols were approved by the Chinese Committee for Animal Use for Research and Education.

Tissue Preparation

Ten adult male or female tender chickens 65 d of age and weighing of 1.5 to 2.0 kg were purchased from Nanjing Farm product market (Nanjing, Eastern China). The birds were housed for an adaptation period of 3 to 4 d and were then anesthetized with an intraperitoneal injection of ethylurethane at1 g/kg and were fastened onto a stereotaxic frame. A horizontal incision, about 5 cm long, was made in the infrapubic region of the abdominal wall, 2 cm ventrally to the shaft of the pubis. The musculus obliquus externus abdominus, musculus rectus abdominus, musculus transversus abdominus, and the very thin peritoneum were then cut open. The small or large intestine was exposed through this incision. The segment of the small intestine from the duodenojejunal flexure to the vitelline diverticulum was tentatively regarded as the jejunum, and that from the vitelline diverticulum to the ileocecal junction as the ileum. The chickens were deeply anesthetized and were perfused downward through the left common carotid artery with physiological saline, followed by a fixative containing 4% paraformaldehyde in 0.1 *M* phosphate buffer (4°C, pH 7.4). The INR was dissected out separately from the jejunal, ileal, and rectal portions. Tissues were immersed in the same fresh fixative solution for 4 h and then stored overnight in a 20% sucrose solution at 4°C. Frozen serial sections (40 μm) were prepared by cutting in the longitudinal plane of the intestinal nerves and were placed on polylysine-coated glass slides. Every 5 serial sections of juxta-jejunoileum and juxta-rectal portion of the INR were designated as a group, and within each group, the third section was stained with toluidine blue; the second and fourth ones were stained with antisense probes of VIP and SP mRNA, respectively; and the first and last ones were used as controls of ISHH, in which the antisense probes were replaced with sense probes of VIP and SP mRNA.

ISHH

In situ hybridization was performed essentially according to Young et al. (1986) with the following modifications for the detection of VIP and SP mRNA transcripts.

Probe Preparations. For preparation of sense and antisense DIG-labeled RNA probes, fragments of VIP and SP mRNA genes were obtained by reverse transcription-PCR from the total RNA of chicken brains. The PCR-amplified cDNA fragments were subcloned into PGM-T Easy Vectors (Tiangen Biotech Co. Ltd., Peking, China), and then the plasmids were transformed into *Escherichia coli* DH5α and chosen by white-blue plaque selection. Recombinant VIP/PGM-T and SP/PGM-T Easy Vectors that had been identified by sequencing were linearized with the restriction enzymes *Nco*I and *Sal*I, respectively. According to the protocol of the DIG RNA Labeling Kit (SP6/T7, Roche, Nutley, NJ; Lobjois et al., 2004), sense and antisense DIG-labeled RNA probes were produced by SP6 and T7 RNA polymerase, respectively. Both the sense and antisense RNA probes were prepared and confirmed by dot blot hybridization.

The following sense primer was used to amplify a 217-bp segment of chicken VIP mRNA cDNA by PCR: 5′ GTC CTG TCA AAC GCC ACT CT 3′ (U09350: 529–548; X80906: 389–408), and the antisense primer was 5′ AGG TGG CTC AGC AGT TCA TC 3′ (U09350: 745–726; X80906: 605–586). A 217-bp chicken SP cDNA of 217 bp was also obtained by PCR. The sense primer was 5′ GCG AAC GGA CGT CAA GGT 3′ (XM 418674: 1,705–1,722), and the antisense primer was 5′ GGC CAA AGA ACT GCT GGG 3′ (XM418674: 1,921–1,903). Both of the PCR products were analyzed by electrophoresis on 2% agarose gel.

Hybridization. For the in situ hybridization experiments, the custom service provided by Su et al. (1998). Briefly, tissue sections were rinsed 3 times in PBS at pH 7.4 for 5 min, in glycine-PBS at 0.1 mol/L for 5 min, in 0.3% TritonX-100-PBS for 25 min, and were digested with proteinase-K-PBS for 25 min at 37°C, followed by fixing in 4% paraformaldehyde in PBS, treating with triethanolamine-acetic anhydride, and washing. Then sections were prehybridized for 1.5 h and hybridized at 57°C for 18 h in hybridization solution containing DIG-labeled RNA antisense probe at 2 μg/mL. After hybridization, the tissue was subjected to stringent washing in 50% 2 × saline sodium citrate (**SSC**), 1 × SSC, and 0.1 × SSC, at 37°C, for 10 min each. Then the sections were rinsed again in PBS, blocked for 30 min with 1.5% normal goat serum (Jiti Biology Company of Nanjing, China) in PBS, and rinsed in PBS. The sections were

Table 1. Percentages of vasoactive intestinal peptide mRNA-positive neurons in juxta-jejunoileum and juxta-rectal portion of intestinal nerve of Remak (INR)

	Juxta-jejunoileum portion in INR			Juxta-rectal portion of INR		
n	Result of ISHH[1]	Toluidine blue staining	Percentage	Result of ISHH	Toluidine blue staining	Percentage
1	36	47	77	297	324	92
2	98	129	76	54	73	74
3	23	30	77	394	459	86
4	37	43	86	553	712	78
5	23	35	66	306	324	94
6	67	85	79	578	644	90
7	12	27	44	287	375	77
8	134	157	85	418	477	88
9	101	126	80	467	632	74
10	41	53	77	115	178	65
Mean			75			87
SD			12.02			9.63

[1]ISHH = in situ hybridization histochemistry.

then incubated with goat anti-DIG antibody (Roche) at 1:100 to 1:1,000 for 2 h at 37°C. After a rinse in PBS for 10 min, the sections were incubated in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche) complex for 1 to 16 h at room temperature and examined under a standard light microscope.

Statistical Data. The reacted sections were examined with a BH-2 microscope (Olympus, Tokyo, Japan). The positive cells distributed in corresponding campus visualize in the 3 serial sections that had been hybridized with VIP, SP antisense probes, and stained with toluidine blue, respectively, were counted. The numbers of VIP and SP-positive cells and toluidine blue-stained cells observed at each location were expressed as the mean ± SE (n = 10 chickens) in Tables 1 and 2.

RESULTS

Certified by dot blot hybridization, the sense and antisense RNA probes were prepared and used in the ISHH.

The Distribution of VIP mRNA in the Chicken INR

Many VIP mRNA-positive neurons that were labeled by VIP mRNA antisense probes were distributed in the ganglion and nerve trunk of the INR, and the neurons were large size and were round or oval. Some of the INR fibers were weakly positive (Figure 1). The percentage of VIP mRNA-positive neurons in the juxta-jejunoileum was 75 and 87% in the juxta-rectal portions of the INR, which were shown in Table1.

The Distribution of SP mRNA in the Chicken INR

Most SP mRNA-positive cells were oval or polygonal, and the labeled neurons were distributed in layers or small groups in the ganglion and sporadically in the nerve trunks between ganglions (Figure 2). The proportion of positive cells among the total neurons was 83% in the juxta-jejunoileum portion and 98% in the juxta-rectal portion of INR (Table 2).

DISCUSSION

The pattern of regulation of the avian INR in the intestine is the same as that of prevertebral ganglia innervating the intestine in mammals (Fontaine-Perus et al., 1981). Previous studies of the distributions of peptide transmitters in the INR used only immunohistochemical methods at the protein level. Because of differences among the antibodies, many of the results were contradictory; therefore, the present study was undertaken. This is the first report of the distributions of peptide transmitters in the avian INR at the gene level.

The VIP is an important noncholinergic and nonadrenergic peptide transmitter in the digestive tract. In the avian intestinal wall, the VIP is distributed in the inhibitory neurons of the myenteric nerve plexus and the secretomotors of the submucous nerve plexus. These VIP neurons inhibit the movements of the enteric smooth muscles and promote the secretion of glandular organs by acting directly on the gland or indirectly on other submucous neurons (Epstein and Poulsen, 1991; Suzuki et al., 1996). With regard to the existence of VIP neurosomes, the cell bodies of the neurons, in the chicken INR, there are 2 completely different opinions. Epstein et al. (1983) found no VIP neurons in the INR of the chick embryo in the final stage of incubation, when they used immunohistochemistry. However, in a study with rabbit anti-human antibody, Suzuki et al. (1994) found VIP nerve fibers only in the INR of adult chickens. In contrast, Aisa et al. (1997, 1998) found VIP neurosomes distributed in ganglia and in nerve tracts between the ganglia and the VIP nerve fibers originating from the INR and innervating the intestinal tract in 2-d-old chickens. At the same time, they found only VIP nerve fibers distributed in the walls of the rectum and cloaca and found no neurosomes. In light of

Table 2. Percentage of substance P mRNA-positive neurons in the juxta-jejunoileum and juxta-rectal portion of intestinal nerve of Remak (INR)

	Juxta-jejunoileum portion in INR			Juxta-rectal portion of INR		
n	Result of ISHH[1]	Toluidine blue staining	Percentage	Result of ISHH	Toluidine blue staining	Percentage
1	33	47	70	333	324	103
2	126	129	98	59	73	81
3	23	30	77	417	459	91
4	39	43	91	703	712	99
5	17	35	49	321	324	99
6	79	85	93	643	644	100
7	24	27	89	377	375	101
8	128	157	82	475	477	100
9	113	126	90	673	632	106
10	33	53	93	177	178	99
Mean			83			98
SD			14.66			7.04

[1]ISHH = in situ hybridization histochemistry.

these results, it was concluded that VIP nerve fibers in the rectum and cloaca originated from VIP neurons of the INR. In the present study, numerous VIP mRNA-positive neurons were observed in all the INR of adult chicken, and the percentages of VIP mRNA-positive neurons were 75 and 85% in the juxta-jejunoileum INR and the juxta-rectal INR, respectively.

With regard to the distribution of SP in the chicken INR, the immunohistochemical study of Suzuki et al. (1994) confirmed that there were abundant SP neurosomes widely distributed in the ganglias of the jejunoileum and that no SP neurosomes were found in the rectal portion of the INR, but there were many SP-positive nerve fibers distributed in both the jejunoileum and rectal portions of the INR. Lunam (1991) preincubated tissue with colchicine to block axonal transport, to prevent movement of peptides from the cyton to the nervous process, and enhance the detection of SP-positive neurosomes. He found that there were mostly SP-positive nerve fibers but no neurosomes in the INR. However, Aisa et al. (1998) used immunohistochemical methods and found that SP-positive neurosomes were present in the whole INR, mainly concentrated in the ganglia. In the present study, abundant SP mRNA-pos-

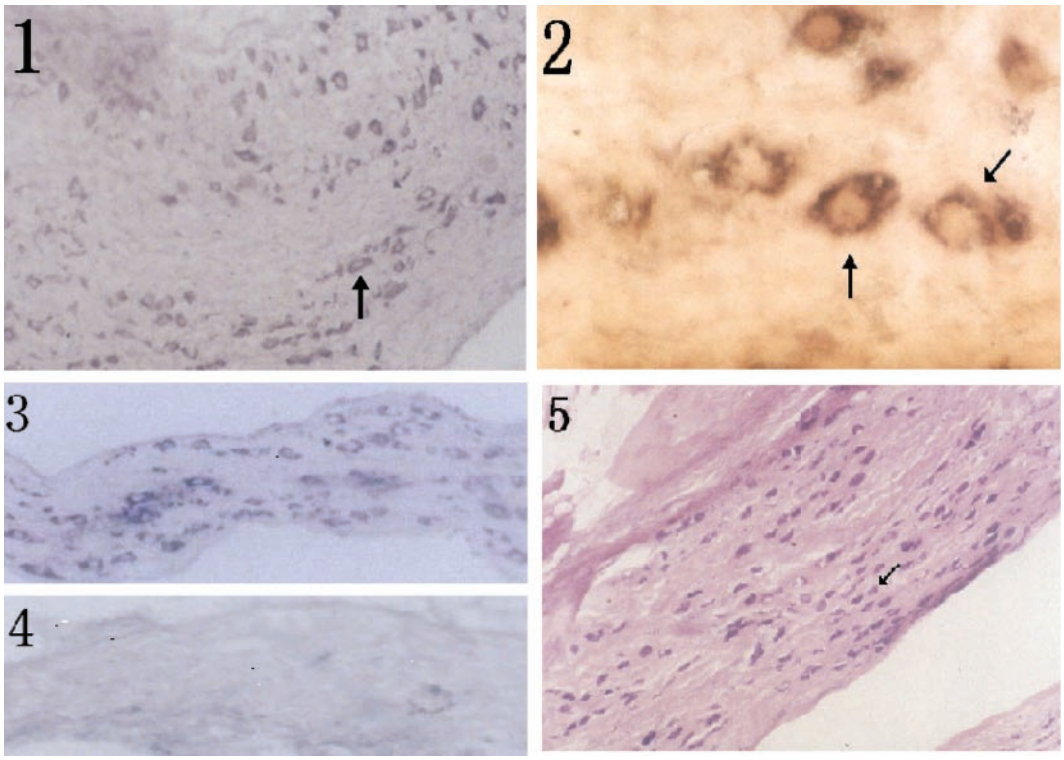

Figure 1. Distribution of vasoactive intestinal peptide mRNA in the intestinal nerve of Remak (INR) of the chicken. 1) Reaction of in situ hybridization histochemistry (ISHH) on the ganglion of juxta-rectal portion of INR × 100. 2) Reaction of ISHH on the ganglion of juxta-rectal portion of INR × 400. 3) Reaction of ISHH on the ganglion of juxta-jejunoileum portion of INR × 100. 4) Negative control × 100. 5) Toluidine blue and eosin staining of ganglion of juxta-rectal portion of INR × 100. Arrows: positive neurons.

itive neurons were found in the whole INR; the proportions of SP mRNA-positive neurosomes were 83 and 98%, respectively, in the juxta-jejunoileum and juxta-rectal portions of the INR. This result was very similar to that of Aisa et al. (1998).

With regard to the source of VIP and SP in the intestinal wall, Fontaine-Perus et al. (1981) demonstrated that part of the VIP and SP nerves in the intestinal wall represented intrinsic innervation. Moreover, they found that some VIP and SP neurons in other auto-

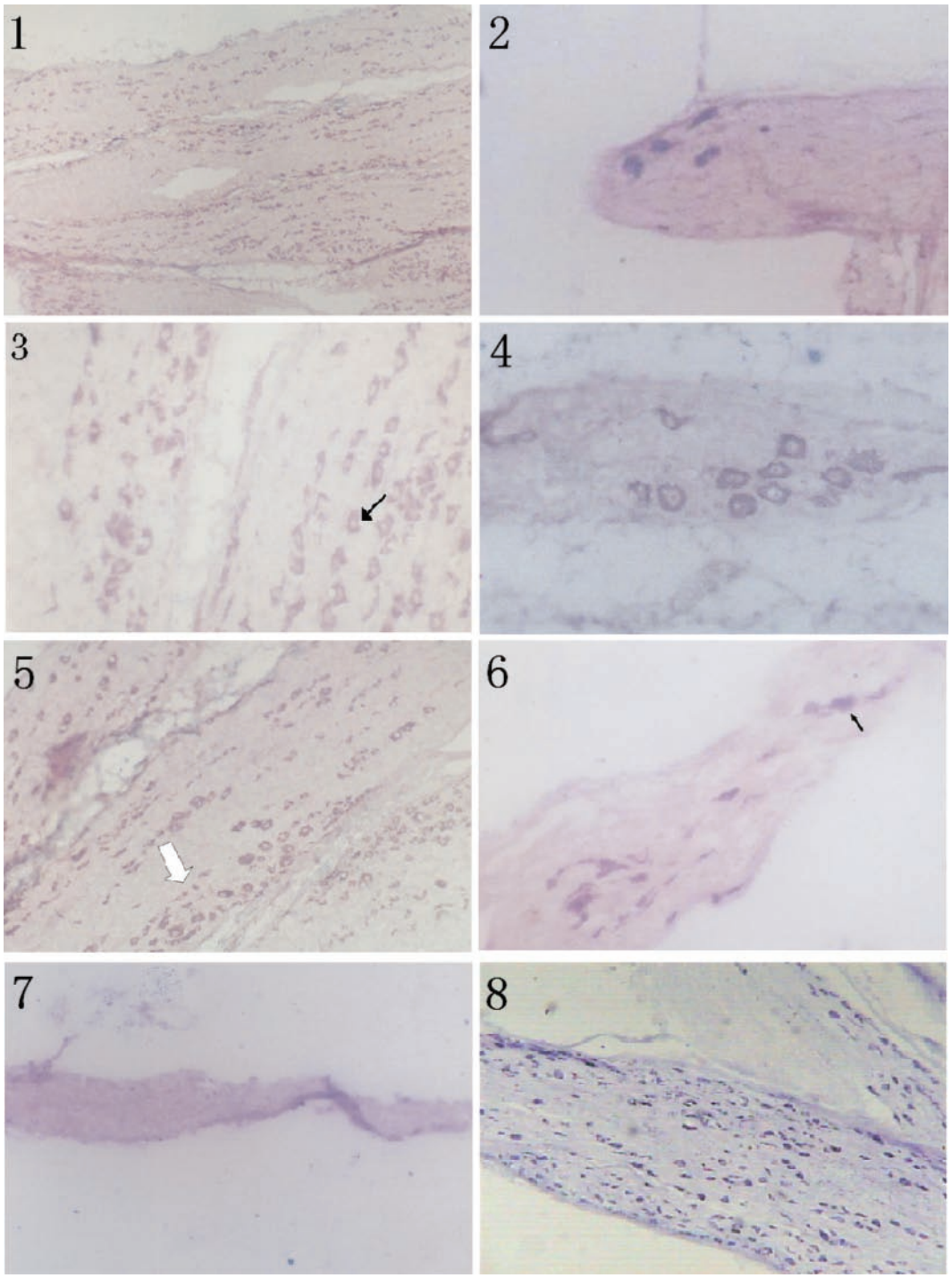

Figure 2. Distribution of substance P mRNA in the intestinal nerve of Remak (INR) of the chicken. 1) Reaction of in situ hybridization histochemistry (ISHH) on the ganglion of juxta-rectal portion of INR, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche, Nutley, NJ) staining × 40. 2) Reaction of ISHH on the ganglion of juxta-jejunoileum portion of INR, NBT/BCIP staining × 200. 3) Reaction of ISHH on the ganglion of juxta-rectal portion of INR, NBT/BCIP staining × 200. 4, 5) Reaction of ISHH on the ganglion of juxta-rectal portion of INR, NBT/BCIP staining × 100. 6) Reaction of ISHH on the nerve bundle between ganglions of juxta-rectal portion of INR, NBT/BCIP staining × 200. 7) Negative control with sense probe × 200. 8) Toluidine blue staining of ganglion of juxta-rectal portion of INR × 40. Arrows: positive neurons.

nomic ganglia also send nerve fibers to the intestines. The results of the present study found that many VIP and SP mRNA-positive neurons were distributed in the INR, and their proportion in the posterior segment was higher than that in the anterior segment. In light of related studies, we presume that some of these VIP and SP mRNA-positive neurons in the INR are the third source of nerve fibers innervating the intestine.

Interestingly, the regulation of the various functions of VIP and SP was dependent upon the same neuron in the INR. Of these labeled neurons observed in the present study, 75 and 83%, respectively, of VIP mRNA-positive neurons and SP mRNA-positive neurons were distributed in the juxta-jejunoileum portion of the INR, and the corresponding proportions in the juxtarectal portion of the INR were 87 and 98%, respectively. These data suggested that VIP mRNA and SP mRNA may be colocalized in some neurons of the INR (i.e., VIP and SP may be coexpressed in some neurons of the INR). In the present study, we were unable to obtain cells labeled with both riboprobes. Suzuki et al. (1994) used immunohistochemistry and found that methionine enkephalin and somatostatin colocalized in the same neurons in the rectal portion of the INR in proportions of rectum approximately 50%. Thus, coexistence of transmitters may be a universal phenomenon in neurons of the INR.

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