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Effects of Eel Neuropeptide Y on Ion Transport Across the Seawater Eel Intestine

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ABSTRACT—A neuropeptide Y (eNPY) was isolated from the intestinal extract of eels. This peptide enhanced significantly the serosa-negative transepithelial potential difference (PD) and short-circuit current (I_{sc}) across the intestine of the seawater eel after pretreatment with isobutylmethylxanthine, serotonin and methacholine. The effects of eNPY on the I_{sc} were concentration-dependent with a threshold concentration of 3×10^{-9} M and a maximal effect at 3×10^{-7} M. Similar concentration-responce curve was obtained by porcine peptide YY (pPYY). Since 9 amino acid residues are replaced in the pPYY, this result indicates that these substitutions do not change the potency and the efficacy. These stimulatory actions of eNPY were not blocked by tetrodotoxin, an inhibitor of neural firing, or yohimbine, an α_2-adrenoceptor antagonist, indicating that eNPY acts without enteric neural firing or catecholamine release. When eNPY and adrenaline (AD) were applied simultaneously, the effects were additive only at lower dosage (3×10^{-8} M for eNPY, 3×10^{-8} M for AD), but not at high dosage (10^{-6} M eNPY, 10^{-7} M AD). The ceiling effect at high dosage suggests that these two regulators act through common signal transduction systems and affect the Na^+-K^+-Cl^- cotransport system, since both effects were completely blocked by bumetanide, a specific inhibitor of Na^+-K^+-Cl^- cotransporter.

INTRODUCTION

Regulation of ion and water transport across the intestine is essential for survival in sea water and fresh water of euryhaline teleosts such as eel and flounder. However, only a few regulators have been proposed in the intestinal ion transport of these teleosts. As an inhibitor, vasoactive intestinal peptide (O'Grady and Wolters, 1990; Uesaka *et al.*, 1995) and atrial natriuretic peptide (Ando *et al.*, 1992; O'Grady *et al.*, 1985) have been proposed in both eel and flounder. In the eel intestine, acetylcholine and serotonin (5-HT) (Mori and Ando, 1991) have also been reported to reduce NaCl absorption. In contrast, as an enhancer, only catecholamines have been reported to enhance the short-circuit current (I_{sc}) from serosa to mucosa across the intestine of the seawater eel (Ando and Kondo, 1993; Ando and Omura, 1993). The I_{sc} is due to active Cl^- transport from mucosa to serosa across the intestine of these fish (Ando *et al.*, 1975; Huang and Chen, 1971).

To find out another endogenous regulators in the eel intestine, we tried to isolate peptides from the intestinal extract, and found previously that various somatostatin-related peptides enhanced the I_{sc}, accompanied by an enhancement in NaCl and water absorptions (Uesaka *et al.*, 1994a, b). During the isolation process, other bioactive fractions which enhance the I_{sc} were also obtained.

The present study was aimed to characterize one of such bioactive peptides. A neuropeptide Y-related peptide (eNPY) was isolated as such a regulatory peptide. In the present study, the effects of eNPY on intestinal ion transport were also examined, in relation to the adrenaline (AD) actions.

MATERIALS AND METHODS

Intestinal extraction and peptide purification

A boiled-water extraction of 100 eel intestine (199 g wet weight), from which pancreas was removed, was prepared by the method described previously (Uesaka *et al.*, 1994b). The extract enhanced the transepithelial potential difference (PD) across the intestine of the seawater eel. The bioactive material was isolated from the extract with Sep-Pak C18 cartridges (Millipore, Milford, MA). The retained material was further purified by reverse-phase (C8P-50, Asahi Chemical, Kanagawa) and cation-exchange (CM-5PW, Tosoh, Tokyo) HPLC. In all reverse-phase steps, elution was performed with a linear gradient of 0-70% acetonitrile containing 5% 2-propanol and 0.1% trifluoroacetic acid (TFA) for 70 min. In cation-exchange step, elution was performed with a linear gradient of 0-0.5 M NaCl in 10% ethanol and 20 mM phosphate buffer (pH 6.7) for 50 min. The final purification

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was performed with HPLC on C18 column (ODS-120T, Tosoh), eluted isocratically using 26% acetonitrile containing 5% 2-propanol and 0.1% TFA.

Peptide structural analysis

For amino acid analysis, a small portion of the purified peptide was dried down in tube. The tube was placed into a hydrolysis chamber with 6 N HCl containing 1% phenol and heated to 110°C for 20 hr. After cooling, the amino acids were analyzed on PICO-TAG amino acid analyzer (Millipore). Amino acid sequence of the purified peptide was determined by automated Edman degradation with a gas-phase sequencer (PPSQ-10, Shimadzu, Kyoto).

Measurement of electrical parameters

The cultured Japanese eels, *Anguilla japonica*, weighing about 210 g, were kept in sea water (20°C) for more than 1 week. After decapitation, the posterior part of the intestine was excised rapidly and the outer muscle layers were stripped off according to previous methods (Ando and Kobayashi, 1978). The mucosal segment was opened and mounted on an Ussing chamber with an exposed area of 0.5 cm^2. The intestinal sheet was bathed in Krebs bicarbonate Ringer's solutions consisting of (mM): 118.5 NaCl, 4.7 KCl, 3.0 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$, 5 glucose and 5 alanine. The bathing solution (3 ml each) was bubbled with 95% O_2-5% CO_2 gas mixture (pH 7.4) at 20°C. Details are described elsewhere (Uesaka *et al.*, 1994b)

The PD was recorded through a pair of calomel electrodes with a polyrecorder (EPR-151A, Toa, Tokyo) as a serosal potential with respect to the mucosa. Rectangular pulses, 30 μA for 500 msec, were applied across the tissue every 5 min. The tissue resistance (R_t) was calculated from the deflection of the PD ($R_t = \Delta PD / 30\ \mu A$). The short-circuit current (I_{sc}) was obtained from the ratio of PD to R_t ($I_{sc} = PD / R_t$). Intestinal extract and synthesized peptides were applied to the serosal bathing medium after pretreatment with isobutylmethylxanthine (IBMX), serotonin (5-HT) and methacholine (MCh).

Reagents

Eel neuropeptide Y (eNPY) for this study was synthesized automatically using a N^{α}-9-fluorenylmethoxycarbonyl protection strategy (PSSM-8, Shimadzu, Kyoto) and purified by HPLC on C_{18} column (TSKgel ODS-120T). Acetyl-β-methylcholine bromide (MCh), (−)adrenaline (AD), 5-hydroxytryptamine creatine sulfate (5-HT), 3-isobutyl-1-methylxanthine (IBMX), porcine peptide YY (pPYY), tetrodotoxin (TTX) and yohimbine HCl were purchased from Sigma Chemical (St. Louis, MO). Bumetanide was kindly gifted from Sankyo Co. (Tokyo).

Statistical analysis

Data are reported as mean±S.E.M.; *n* represents the number of preparations. The statistical significance of difference between means

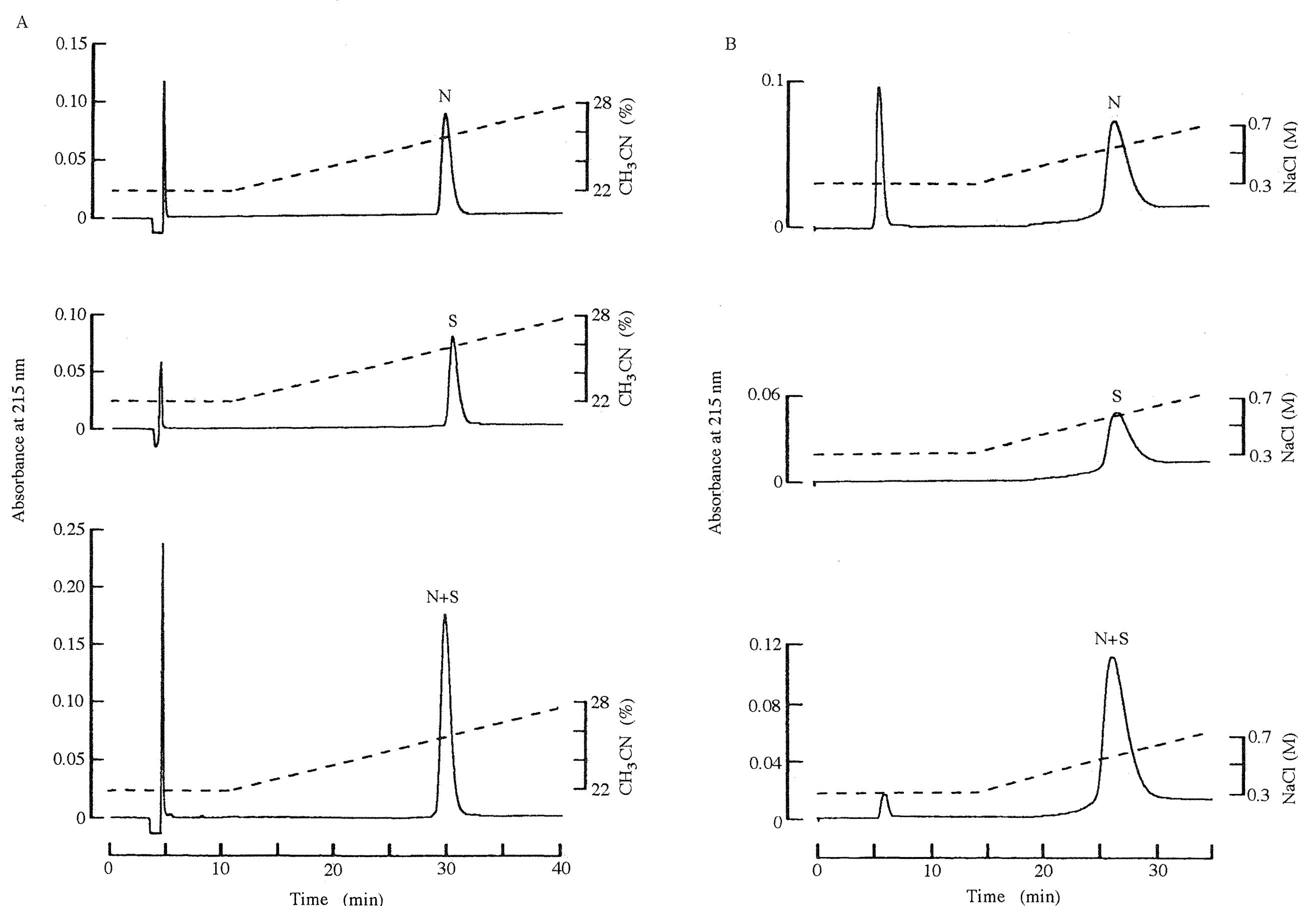

Fig. 1. Comparison of chemical characteristics between native (N) and synthetic (S) eel neuropeptide Y (eNPY). (A) Reverse-phase chromatograms of N, S and N+S. Each peptide and thier mixture were eluted with a 30-min linear gradient of 22%-28% acetonitrile in 5% 2-propanol and 0.1% TFA at a flow rate of 0.5 ml/min. (B) Cation-exchange chromatograms with CM-5PW column. N, S and N+S were eluted with a 20-min linear gradient of 0.3-0.7 M NaCl in 10% 2-propanol and 20 mM phosphate buffer (pH 6.7). Flow rate was 0.5 ml/min.

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was examined with a Mann-Whitney's U-test. The null hypothesis was rejected for $P<0.05$.

RESULTS

Isolation and characterization of eel neuropeptide Y

A bioactive material was isolated from the intestinal extract. The purified substance enhanced the serosa-negative PD across the seawater eel intestine after pretreatment with IBMX, 5-HT and MCh. Without such pretreatment, its effects varied among preparations (data not shown).

By sequence analysis, the bioactive substance was found to be a peptide with the following amino acid sequence; Tyr-Pro-Pro-Lys-Pro-Glu-Asn-Pro-Gly-Glu-Asp-Ala-Ser-Pro-Glu-Glu-Gln-Ala-Lys-Tyr-Tyr-Thr-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr. The quantitative amino acid analysis gave the following amino acid composition in this peptide (residues/mol peptide): Asx 3.0; Glx 5.5; Ser 1.3; Gly 1.4; His 1.3; Arg 2.9; Thr 2.6; Ala 3.2; Pro 5.4; Tyr 4.7; Ile 2.0; Leu 2.0; Lys 1.9. This confirms the primary structure obtained by the sequence analysis. On the basis of these results, the isolation yield from 199 g of guts was 15.5 μg (3.6 nmol). However, the same sequence had already been reported as eel NPY-related peptide (eNPY) by Conlon *et al.* (1991). They isolated it from the pancreas of American eels. Since the pancreas eNPY is α-amidated, we synthesized C-terminally amidated eNPY. The synthesized peptide was eluted at the same retention time of the native eNPY both in a reverse-phase and a cation-exchange HPLC (Fig. 1). Since C-terminally free eNPY elutes more fast than the amidated eNPY on the cation-exchange HPLC, this indicates that the native eNPY is amidated C-terminally. Therefore, the following experiments were all performed by using the synthesized C-terminally amidated eNPY.

Effects on the eel intestine

Figure 2A shows the effects of eNPY in the presence of TTX. Even after blocking neural firing with TTX (10^{-6} M), eNPY (3×10^{-7} M) enhanced the serosa-negative PD and I_{sc} significantly (ΔPD=4.6±0.6 mV, ΔI_{sc}=25.5±5.9 μA/cm², n=5, $P<0.05$). But the effect on R_t was not significant (ΔR_t=11.7±4.8 Ω cm²). As similar enhancements in the PD and I_{sc} were observed after AD in this preparation (Ando and Omura, 1993), we examined a possibility that eNPY acts through AD release. However, as shown in Fig. 2B, even after blocking AD (10^{-8} M) actions completely with yohimbine (5×10^{-6} M), an α_2-adrenoceptor antagonist, eNPY (3×10^{-7} M) enhanced the PD and I_{sc} significantly (ΔPD=6.1±1.1 mV, ΔI_{sc}=38.9±7.6 μA/cm², n=5, $P<0.05$) but not significantly in case of R_t (ΔR_t=36.7±14.9 Ω cm²). Table 1 summarizes the effects of eNPY on these three electrical parameters after pretreatment with IBMX, 5-HT and MCh.

The stimulatory effects of eNPY on I_{sc} were concentration-dependent, with a threshold concentration of 3×10^{-9} M and a maximal effect at 3×10^{-7} M (Fig. 3). In the next experiment, effect of porcine PYY (pPYY), 75% identity to eNPY, was compared with that of eNPY. As shown in Fig. 3, the concentration-response curve of pPYY were almost identical to that of eNPY.

When bumetanide (10^{-6} M), a specific inhibitor of Na^+-K^+-Cl^- cotransporter, was added to the mucosal fluid after pretreatment with IBMX, 5-HT and MCh, the serosa-negative PD and I_{sc} were further diminished (−4.8±0.3 to −0.9±0.2 mV for PD (n=5), $P<0.05$, −53.9±7.8 to −11.2±2.7 μA/cm² for I_{sc}

Table 1. Effects of eel neuropeptide Y (eNPY) on the transepithelial potential difference (PD), short-circuit current (I_{sc}) and tisssue resistance (R_t) after pretreatment with IBMX, 5-HT and MCh

	PD (mV)	I_{sc} (μA/cm²)	R_t (Ω cm²)
Control	−9.9 ± 0.8	−90.8 ± 12.5	121.6 ± 14.0
IBMX (10^{-5} M) +5-HT (10^{-6} M) +MCh (10^{-6} M)	−4.0 ± 0.5*	−40.8 ± 7.3*	106.3 ± 7.5
eNPY (3×10^{-7} M)	−8.7 ± 0.8†	−75.6 ± 10.1†	129.6 ± 19.3

Values are mean ± S.E.M., n = 10.
*$P<0.05$ compared with the control (Mann-Whitney's U-test).
†$P<0.05$ compared with those treated with IBMX, 5-HT and MCh (Mann-Whitney's U-test).
IBMX, isobutylmethylxanthine; 5-HT, serotonin; MCh, methacholine.

Table 2. Effects of eNPY after pretreatment with bumetanide. In the absence of bumetanide (control), eNPY enhanced the serosa-negative PD and I_{sc} (denoted by an increase in negativity in ΔPD and ΔI_{sc}), but bumetanide blocked these eNPY actions completely.

Experimental groups	Changes induced by eNPY (10^{-7} M)		
	ΔPD (mV)	ΔI_{sc} (μA/cm²)	ΔR_t (Ω cm²)
Control	−4.2 ± 1.1	−34.2 ± 8.9	26.3 ± 25.2
Bumetanide (10^{-6} M)	−0.5 ± 0.3*	−3.1 ± 2.5*	1.3 ± 32.6

Values are mean ± S.E.M., n=5.
*$P<0.05$ compared with the control (Mann-Whitney's U-test).

Table 3. Comparison of the effects of eNPY and AD on the I_{sc} across the seawater eel intestine. All values are expressed as an increase in negativity of the I_{sc} after eNPY, AD or their mixture.

Treatment	Changes in I_{sc} (μA/cm²)
High dosage	
eNPY (10^{-6} M)	−29.2 ± 9.4
AD (10^{-7} M)	−35.0 ± 9.6
eNPY (10^{-6} M) +AD (10^{-7} M)	−40.9 ± 9.6
Low dosage	
eNPY (3×10^{-8} M)	−8.9 ± 1.5
AD (3×10^{-9} M)	−17.3 ± 3.8
eNPY (3×10^{-8} M) +AD (3×10^{-9} M)	−33.4 ± 4.0*†

Values are mean ± S.E.M., n=5.
*$P<0.05$ compared with eNPY alone (Mann-Whitney's U-test).
†$P<0.05$ compared with AD alone (Mann-Whitney's U-test).

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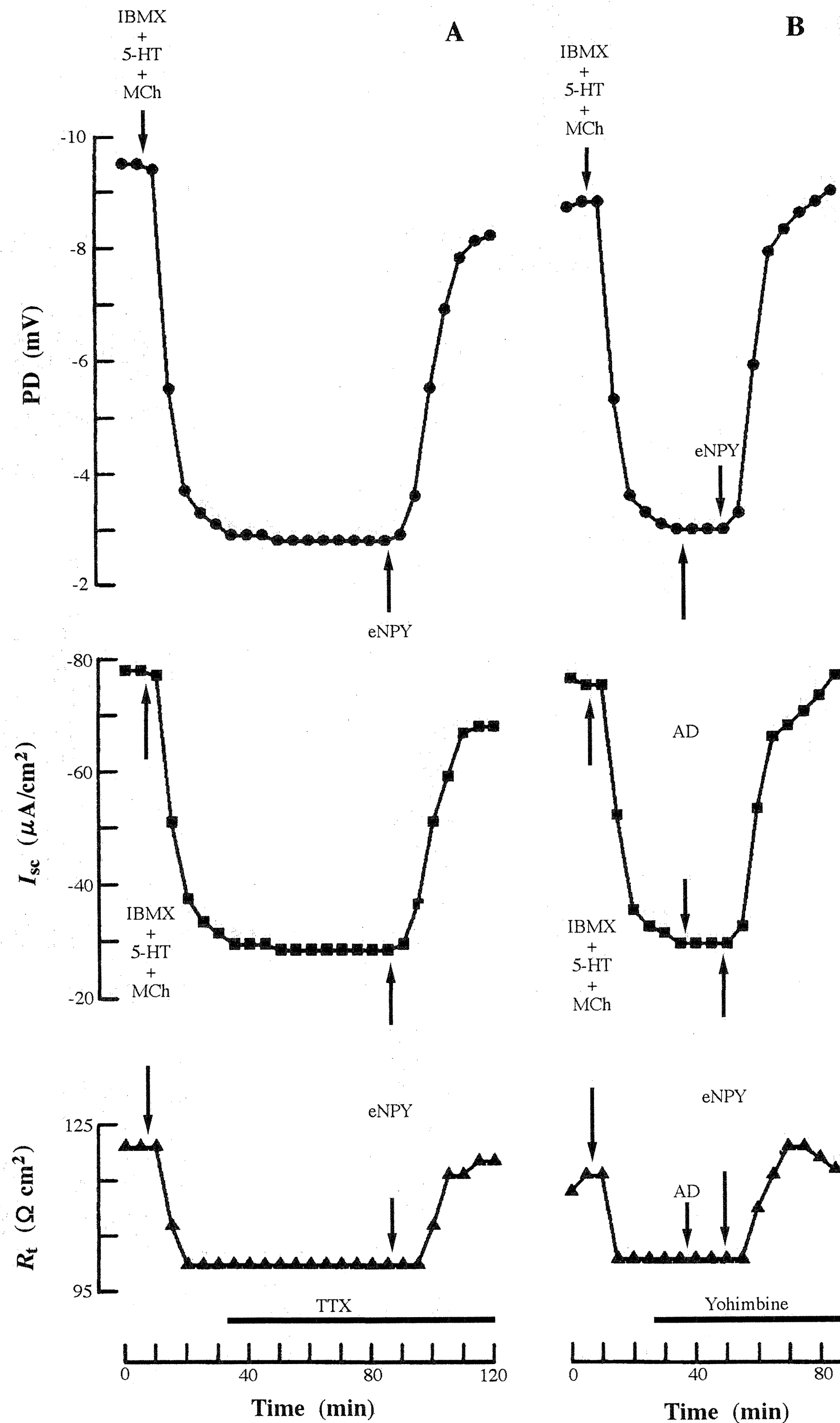

Fig. 2. Independent action of eNPY from neural firing and catecholamine release. (A) Effects of eNPY on the PD (●), I_{sc} (■) and R_t (▲) in the presence of tetrodotoxin (TTX). At the first arrows, IBMX (10^{-6} M), 5-HT (10^{-6} M) and MCh (10^{-6} M) were added to the serosal fluid. After pretreatment with TTX (10^{-6} M, horizontal bar), eNPY (3×10^{-7} M) was added to the serosal bathing medium (second arrows). (B) Effects of eNPY on the PD (●), I_{sc} (■) and R_t (▲) after pretreatment with yohimbine. In the presence of yohimbine (5×10^{-6} M, horizontal bar), AD (10^{-8} M) was added to the serosal medium (second arrows), and eNPY (3×10^{-7} M) at the third arrows. Tracing is representative of four experiments.

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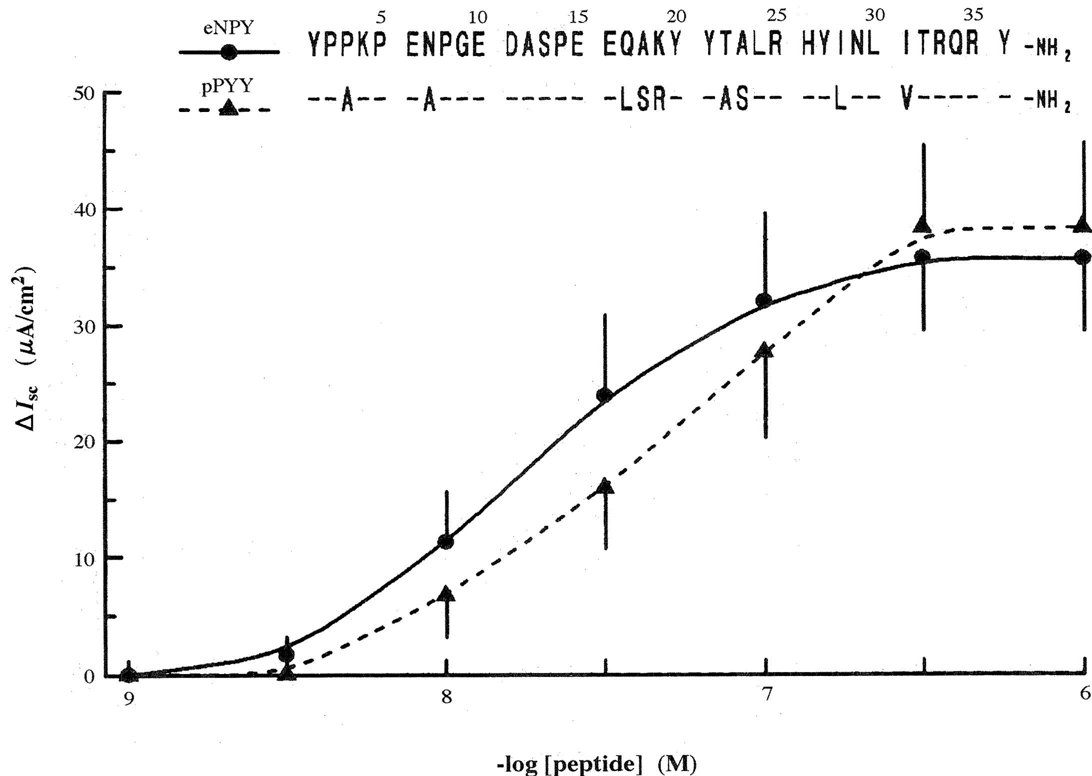

Fig. 3. Concentration-response curve for the effect of eNPY on the I_{sc} across the seawater eel intestine. The increase in the I_{sc} (ΔI_{sc}) after addition of eNPY (●) was plotted against concentrations (on a logarithmic scale). For comparison, effect of porcine peptide YY (pPYY) was also plotted (▲). Each point and vertical bar indicate the mean value and S.E.M. (n=5). Primary structures of these peptides are shown above with a single letter code.

(n=5), P<0.05). In the presence of bumetanide (10^{-6} M), the effects of eNPY were completely blocked (Table 2). AD actions were also blocked by mucosal bumetanide (data not shown).

Interaction between eNPY and AD

As shown in Table 3, eNPY and AD enhanced the I_{sc} similarly. When the supramaximal doses of eNPY and AD were applied simultaneously, the combined effect was not significantly different from that of eNPY alone or AD alone. On the other hand, when lower concentrations of eNPY (3×10^{-8} M) and AD (3×10^{-9} M) were applied simultaneously, the enhancement in I_{sc} was significantly greater (P<0.05) than that induced by eNPY alone or AD alone, i.e. both effects were additive.

DISCUSSION

The present study is the first report to show the effect of eNPY in an homologous system. Eel NPY enhanced the I_{sc} across the intestine in a concentration-depended manner (Fig. 3). Since the I_{sc} is due to active Cl^- transport from mucosa to serosa (Ando and Utida, 1986; Ando *et al.*, 1975) and water follows this transport (Ando, 1975; Ando *et al.*, 1986, 1992; Mori and Ando, 1991; Uesaka *et al.*, 1994b), Cl^- and water absorption will be enhanced by eNPY. The present study also demonstrates that replacement of Pro[3], Asn[7], Glu[17], Ala[18], Lys[19], Thr[22], Ala[23], Ile[28], Ile[31] in the eNPY with Ala[3], Ala[7], Leu[17], Ser[18], Arg[19], Ala[22], Ser[23], Leu[28], Val[31], respectively, does not change the potency and the efficacy, since eNPY and pPYY had similar effect on the I_{sc} (Fig. 3).

Similar enhancement in I_{sc} is also induced by AD (Table 3) (Ando and Omura, 1993). However, the effect of eNPY is not due to AD release, because the eNPY actions were not blocked by yohimbine (Fig. 2), a potent antagonist for AD action in the seawater eel intestine (Ando and Omura, 1993). In addition, the effects of eNPY were not blocked by TTX (Fig. 2), suggesting that the peptide does not act on neurons. Together, eNPY might act directly on the intestinal epithelium.

Although eNPY and AD had similar effect on the I_{sc}, the dose-response curve was more steep in case of AD; threshold concentration of 10^{-9} M and a maximal effect at 3×10^{-8} M (Ando and Omura, 1993). When eNPY and AD were applied simultaneously, the effects were additive only at low dosage, but not at high dosage (Table 3). If eNPY and AD act through a common signal transduction pathway such as cAMP, these results can be explained. Although details of such signal

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transduction pathways are not clear yet in case of eNPY and AD, both regulators may enhance the Na^+-K^+-Cl^- cotransport finally, since the effects of eNPY and AD were completely blocked by bumetanide, a specific inhibitor of the Na^+-K^+-Cl^- cotransporter. In mammals, coexistence of NPY and catecholamines have been observed in some sympathetic neurons (Wahlestedt *et al.*, 1986; Wiley and Owyang, 1987). If eNPY coexists with AD in the enteric nervous system of the eel, it will accelerate AD action and stimulate the Cl^- absorption at lower concentrations, though a possibility that circulating AD regulates the Cl^- absorption hormonally is still remained.

The experimental condition used in the present study was determined after several trials to obtain a constant response (Ando and Omura, 1993). Under such condition, eNPY (present study), somatostatins (Uesaka *et al.*, 1994a, b; Uesaka *et al.*, 1995) and catecholamines (Ando and Kondo, 1993; Ando and Omura, 1993) enhance the I_{sc} in a concentration-dependent manner without exceptions. Probably these regulators may antagonize the inhibitory effects of 5-HT or ACh.

Although eNPY enhances Cl^- transport across the seawater eel intestine, mammalian NPY inhibits Cl^- transport across the rat intestine (Cox *et al.*, 1988). The discrepancy of the effect of NPY may be due to different characteristics between these preparations. Both tissues contain the loop-diuretic sensitive Na^+-K^+-Cl^- cotransporter. However, the localization of the transporter is opposite; on the brushborder membrane of the epithelium in the seawater eel (Ando and Utida, 1986) *vs* on the basolateral membrane in mammals (see Ref. Anderson *et al.*, 1992). Recently, it is clarified from cDNA cloning of various Na^+-K^+-Cl^- cotransporters that molecular structure of these cotransporters varies considerably depending on their localization, apical or basolateral (Soybel *et al.*, 1995).

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