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Local Synthesis of Natriuretic Peptides in the Eel Intestine

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The intestine is a major osmoregulatory organ in euryhaline fishes which allows them to survive in the sea, and natriuretic peptides have been implicated in regulation of transmural transport. Atrial (ANP) and ventricular natriuretic peptide (VNP) were identified in eel intestine. Elution profiles of ANP and VNP from high-performance liquid chromatography (HPLC) were determined by radioimmunoassay using highly specific antisera. Elution times of immunoreactive ANP and VNP in HPLC were identical to those of authentic peptide standards and were consistent with the relative molecular masses of these peptides. Tissue localization of ANP and VNP was accomplished by fluorescence immunohistochemistry. Immunoreactive cells were observed in the epithelium of anterior, middle, and posterior regions of intestine. Reverse transcription of mRNA isolated from intestine and subsequent polymerase chain reaction amplification yielded appropriate-size products consistent with ANP and VNP expression. Together, these data show that natriuretic peptides are synthesized locally in eel intestine, rather than trapped from the circulation.

The alimentary tract is both the source of, and target for, a large number of peptide hormones which regulate intestinal function. The natriuretic peptides, in addition to their widespread roles as natriuretic and diuretic factors, are inhibitors of transmural salt absorption across the intestine of vertebrates (1) and, may be particularly important in fishes where salt absorption is essential to survival (2,3). Interestingly, in fishes, natriuretic peptides are 1000-fold more potent in their action than other inhibitory drugs such as acetylcholine, histamine and serotonin (2-4). The earliest ultrastructural and physiological studies focused on cardiac production of natriuretic peptides (5-8) but more recently, isolated studies have yielded evidence for enteric, and other extracardiac, production of natriuretic peptides (9-14). Evidence for intestinal synthesis and secretion signals the possible involvement of natriuretic peptides in local, paracrine and autocrine regulation of this tissue. In this study, we examined using chromatography, radioimmunoassay, immunohistochemistry, and reverse transcription-polymerase chain reaction the enteric production in the Japanese eel of two particular natriuretic peptides, atrial natriuretic peptide (ANP) and ventricular natriuretic peptide (VNP), both of which are potent inhibitors of salt transport across the intestine of this species (3).

MATERIALS AND METHODS

Materials. Japanese eels (*Anguilla japonica*) weighing 180-200 g were obtained from commercial sources. They were acclimated in the laboratory to freshwater and to natural seawater at 18 °C for at least two weeks before use. Fish were anesthetized in 0.15% 3-aminobenzoic acid ethyl ester (methanesulfonate salt) in fresh water or seawater (buffered with $NaHCO_3$ to neutral pH), and decapitated prior to removal of the intestine. The intestine was placed in chilled eel Ringer solution (ER, composition (in $mmol \cdot l^{-1}$): Na^+, 143.5; K^+, 5.9; Ca^{2+}, 3.0; Mg^{2+}, 1.2; Cl^-, 149.2; SO_4^{2-}, 1.2; HCO_3^-, 5.0; $H_2PO_4^-$, 1.2; pH, 7.4), opened along a mesenteric border, and thoroughly rinsed with ER. The posterior intestine was defined as that segment caudad to the well-developed muscular sphincter in the terminal gut. The anterior and middle segments were defined in this study to be the cranial and caudal halves, respectively, of the intestine between the point of attachment of the stomach, and the muscular sphincter.

Extraction, chromatography, and radioimmunoassay (RIA). Procedures for extraction and chromatography followed those published earlier by Takei and coworkers for the isolation and characterization of individual eel (e) NPs and of the 14 kDa proANP and proVNP from the heart and brain of Japanese eel (15-17). Extracts were prepared from mucosal scrapings of anterior, middle and posterior intestinal segments from 3 freshwater-adapted (FW) and from 3 seawater-adapted (SW) eels; mucosal scrapings excluded the muscularis externa and serosal layers. After measurement of wet mass, intestinal scrapings were boiled (100 °C, 5 min) in 5-10 volumes of distilled

The cDNA cloning of eel ANP which is referenced in this report is now in publication as the following: Takei, Y., Ueki, M., Takahashi, A., and Nishizawa, T. (1997) Cloning, sequence analysis, tissue-specific expression and, prohormone isolation of eel atrial natriuretic peptide. *Zool. Sci.*, in press.

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water, cooled, and, following addition of acetic acid (AcOH) to 1 M, were Polytron homogenized and centrifuged; the supernatant was collected and lyophilized. The extracts were next purified on Sep-Pak columns (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA); crude extracts reconstituted in 1 M AcOH were loaded onto Sep-Pak columns, washed with 1M AcOH, eluted with 60% acetonitrile (CH_3CN), and lyophilized.

The Sep-Pak column-purified tissue extracts were subsequently subjected to analysis by gel permeation (GP-) and reversed-phase high-performance liquid chromatography (RP-HPLC). GP-HPLC was performed on a Pharmacia Biotech Superdex Peptide HR 10/30 column (10 × 300 mm; eluent: 30% CH_3CN with 0.1% trifluoroacetic acid (TFA); flow rate: 0.5 ml/min; detection: OD_{220nm}). This gel permeation system was calibrated using ribonuclease A (13.7 kDa, elution time = 18 min) and synthetic eel natriuretic peptides (eANP and eVNP; obtained from the Peptide Institute, Osaka, Japan); the void volume was 8 ml (elution time = 16 min). RP-HPLC analysis utilized a Tosoh ODS 120T column (0.46 × 25 cm; eluent: 10-60% CH_3CN gradient with 0.1% TFA; flow rate: 1 ml/min; detection: OD_{220nm}). Again, synthetic natriuretic peptides were used for calibration.

ANP and VNP elution profiles from both chromatographic systems were determined by RIAs specific for eANP and eVNP, respectively; these assays have been validated for this homologous eel system (16, 17).

Immunohistochemistry. Standard immunohistochemical technique was applied to Japanese eel intestine for the localization of NP. Freshly dissected tissues were prepared for examination by fixation for 2h in 4% paraformaldehyde in phosphate-buffered saline (PBS). Intestinal segments were prepared as flat sheets and flooded with fixative. Hearts were flushed with fixative by perfusion and submerged in fixative; atrium and ventricle were separated by dissection after fixation was complete. Tissues were washed 3-4 times in 50 mM NH_4Cl to quench unreacted aldehyde groups (aiding subsequent antibody penetration) and were then infiltrated with 30% sucrose before embedding in Tissue-Tek O.C.T. compound (Miles Scientific, Naperville, IL, USA) for frozen sectioning. Tissues were sectioned at 7 μm and thaw-mounted on gelatin-coated glass microscope slides. For staining, sections were washed in PBS with 0.3% Triton X-100, and excess buffer was removed by blotting on absorbent paper. When appropriate (especially in cardiac muscle), sections were bathed in 0.1% $NaBH_4$ for 10 min to reduce autofluorescence. Sections were then incubated in normal goat serum (NGS; 1:10 dilution in PBS) at room temperature for 30 minutes. After blotting, sections received either primary antiserum directed against the desired peptide at the appropriate dilution (anti-eANP, 1:100; eVNP, 1:500), or primary antiserum which had been prereacted with excess antigen, or the sections remain in NGS without primary antiserum. To block nonspecific binding, all antisera contained 3% bovine serum albumin (BSA) and 10^{-10} M of the potentially cross reacting NP species (*e.g.*, the eANP antiserum working solution contained 10^{-10} M eVNP and 10^{-10} M eCNP, another peptide which is expressed in eel brain), and all other reagents contained 1% BSA. After incubation at room temperature (1 h) or at 4°C (overnight), sections were rinsed in PBS, blotted and bathed with a secondary, fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. Slides with sections were rinsed in PBS and coverslips were mounted onto the glass microscope slides with glycerin-PBS (9:1) before viewing and photography with epifluorescence microscopy. Interpretation of immunohistochemical staining was made by comparing the tissue sections which received both primary antiserum and secondary antibody with control sections which received either the preabsorbed primary antiserum or only secondary antibody. Positive reactions were recognized as those in which immunofluorescence associated with specific cell types or processes was quenched by both preabsorption and primary antiserum omission.

Reverse transcription of RNA and polymerase chain reaction (RT-PCR). RT-PCR was performed by the method described in Iwami *et al.* (18) with some modifications. Total RNA of atria and ventricles, and of anterior, middle and posterior intestines of FW eels were prepared with ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan). One μg of RNA from each was reverse transcribed with 200 units of Superscript II (Life Technologies, Gaithersburg, MD, USA) in a 20-μl reaction mixture of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM of all dNTPs in the presence of 0.1 mg Oligotex-dT30 Super (Takara Shuzo, Kyoto and Nippon Roche, Tokyo, Japan). The mixture was incubated for 60 min at 42 °C, boiled for 5 min, and chilled quickly on ice. The Oligotex-dT30-cDNA was separated by centrifugation at 12,000 × *g* for 5 min at 4 °C and rinsed twice with 300 μl of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The washed Oligotex-dT30-cDNA was suspended in a 50-μl solution of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.2 mM of all dNTPs. The solution also contained 1.0 μM of each of two primers; for eANP these were 5′-GATTACGAGGACGCCAAGCC-3′ (nucleotides 183-202) and 5′-CTCCTTTGACATTCCGTAGC-3′ (nucleotides 576-548; U. Ueki, T. Nishizawa and Y. Takei, unpublished data), and for VNP these were 5′-GTCACAGAATGGCAAAGTCT-3′ (nucleotides 52-71) and 5′-CTGGTACCAAAACAGCTGTT-3′ (nucleotides 430-411; 19). Amplification was carried out using 2.5 units of Takara Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) for 40 cycles under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, and extension for 2 min at 72 °C. Ten μl of each RT-PCR reaction mixture was electrophoresed on a 2% agarose gel and immersed in 0.1 μg/ml ethidium bromide solution for 30 min. The PCR products were visualized by UV light and photographed.

RESULTS

Elution profiles from GP-HPLC, detected using RIA, displayed clear peaks in eANP and eVNP immunoreactivity (Figure 1). Elution times for the peaks in immunoreactivity of extracts from anterior, middle, and posterior segments of intestine were coincident, and were identical to those of authentic natriuretic peptide standards. Elution profiles were similar for extracts from all three intestinal segments in both FW and SW eels; data from the three segments were averaged for presentation. eANP immunoreactivity eluted from the column as a major peak (*ca.* 22 min; coincident with authentic eANP) with earlier-eluting activity likely representing the 14 kDa proANP hormone molecule, and later-eluting activity perhaps representing degradative products from intestinal enzymatic digestion. The eVNP elution profiles were, again, similar for the three intestinal segments and for FW and SW eels; data from the three segments were averaged, as noted above. Elution profiles for eVNP immunoreactivity showed more prominent major peaks than eANP, but generally lacked the broad shoulders presumed to be degradation products or variant molecular forms of the hormones.

Peaks in the elution profiles for both eANP and eVNP immunoreactivity from RP-HPLC were characteristically broad and bracketed the elution times of authentic peptide standards. The breadth of the peaks may reflect shifts in elution time due to partial hydrolysis of the peptides or variant molecular forms of the hormones.

The immunoassayable amounts of natriuretic pep-

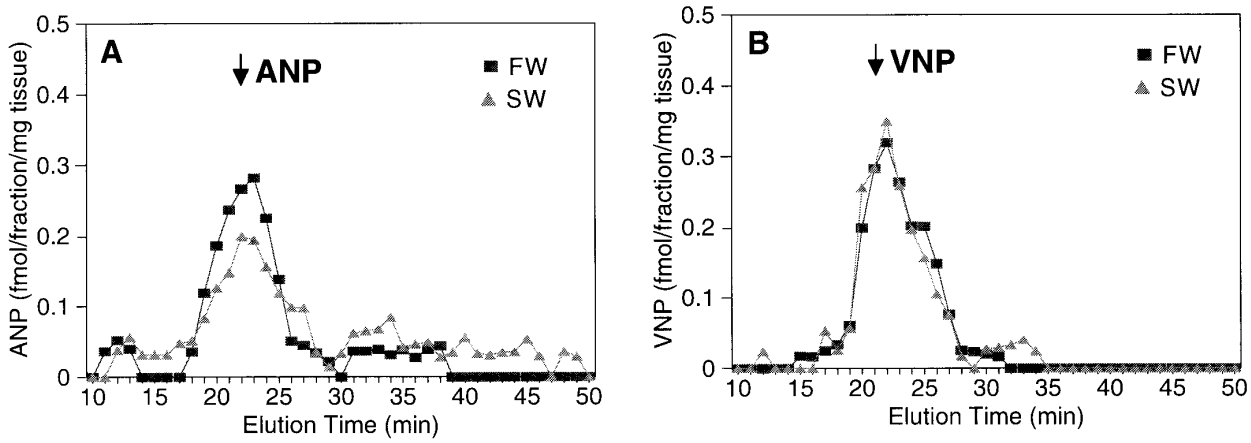

FIG. 1. Gel permeation-HPLC elution profiles for immunoreactive eANP (A) and eVNP (B) in extracts of intestinal mucosa from freshwater- (FW) and seawater-adapted (SW) Japanese eel. Elution times of authentic natriuretic peptide standards are indicated with arrows.

tide recovered from HPLC were generally similar for extracts from all three intestinal segments of FW and SW eels, and for eANP and eVNP, when corrected for initial tissue mass. The amounts of recoverable eANP and eVNP immunoreactivity were in the range of 0.33-2.45 and 1.17-2.15 fmol/mg tissue, respectively.

In immunohistochemistry, cardiac tissue served as positive control and exhibited the expected strong fluorescent signal with immunostaining (Figure 2). Consistent with earlier radioimmunoassay findings (20), atrium exhibited strong fluorescence staining for ANP, whereas ventricle stained for both ANP and VNP. Appropriately, prereaction of antiserum with natriuretic peptides nearly abolished the fluorescent signal.

Immunoreactive eANP and eVNP were detected in diffusely-arranged epithelial cells in all intestinal segments of both FW and SW eels (Figure 3). These cells were relatively more abundant in the epithelium near the bases of villar folds but were visualized near villar tips as well. A nearly-continuous band of subepithelial immunoreactivity was also detected with antisera against eANP. Immunological staining with antisera against eVNP was less intense than that with antisera against eANP.

The Oligotex RT-PCR method efficiently amplified NP mRNA. Atrium and ventricle, as positive controls, generated abundant RT-PCR products of the predicted size for both ANP (385 bp) and VNP (379 bp, Figure 4). From FW eel intestinal tissues, RT-PCR products of the correct sizes were obtained from all three segments using both sets of primers, although product abundance was less than those from heart tissues (Figure 4). ANP RT-PCR product was especially abundant in the anterior intestine.

DISCUSSION

NP-secreting cells are widely distributed throughout the vertebrate body. Recent studies in mammals using immunohistochemical and biochemical techniques have extended the list of ANP-secreting cells to include a number of extracardiac tissues (1, 10) and it is not surprising that the intestine, a rich source of hormones of the gastroenteropancreatic series, also expresses NPs (1, 11-14, 20). Such lists of sources and targets suggest a broader regulatory role than originally conceived and recent reports on the localization of NPs in piscine tissues (1, 16, 20, 21) support the assertion in this vertebrate class, too. The strong inhibition of coupled Na^+-Cl^- absorption by NP is a common finding in teleosts (1, 2).

Delivery of NP through the general systemic circulation from cardiac or other sources integrates the regulation of intestinal ion transport into a global adaptive osmoregulatory response. Paracrine, or even autocrine, secretion of NP by enteric cells in response to local conditions would affect control of intestinal transport on a more restricted anatomical scale, perhaps coupling intestinal function to luminal load. The results of our experiments demonstrate local synthesis to be the source of these chemical messengers, rather than trapping from the circulation. The precise stimulus for paracrine secretion is unknown but presumably could be either osmotic, chemical (electrolyte, nutrient, or neurotransmitter) or mechanical (volume). Earlier reports on the apparent ability of the intestine to taste locally suggest a sensory input pathway to the enteric nervous system; Lennane *et al.* (22, 23) demonstrated greater natriuresis in rabbits and humans following oral delivery of a salt load compared with intravenous delivery of an equivalent salt load. Subsequent ultrastructural studies described "clear" intestinal epithelial cells of the open type which lacked the secretory granules characteristic of endocrine cells and which displayed associations with submucosal enteric neurons (24). These cells may be sensors for the enteric nervous system

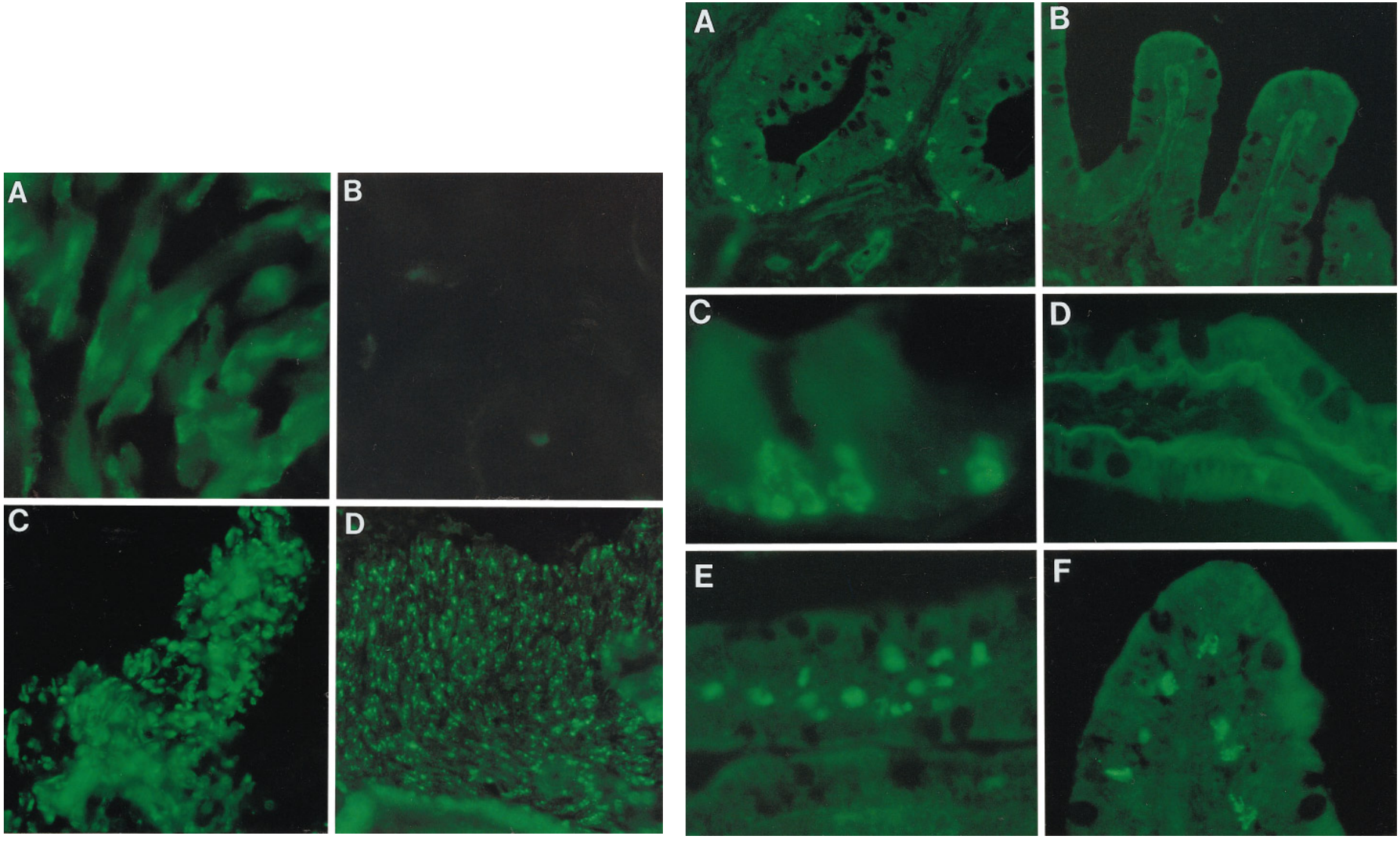

FIG. 2. FITC-immunofluorescent staining for eANP and eVNP of eel heart as positive controls. (**A**) Anti-eANP on atrium (mag ×1000), (**B**) prereacted anti-eANP (mag ×1000), (**C**) anti-eVNP on atrium (mag ×250), and (**D**) anti-eVNP on ventricle (mag ×250).

FIG. 3. FITC-immunofluorescent staining for eANP and eVNP of eel intestine. (**A**) Anti-eANP on anterior intestinal villi (mag ×260), (**B**) prereacted anti-eANP on anterior intestinal villi (mag ×260), (**C**) eANP-positive epithelial cells at base of anterior intestinal villar fold (mag ×1050), (**D**) subepithelial eANP-immunoreactive band in middle intestine (mag ×1050), (**E**) eVNP-immunoreactive cells in mid-villus of anterior intestine (mag ×525), and (**F**) eANP-immunoreactive cells at villar tip of middle intestine (mag ×525).

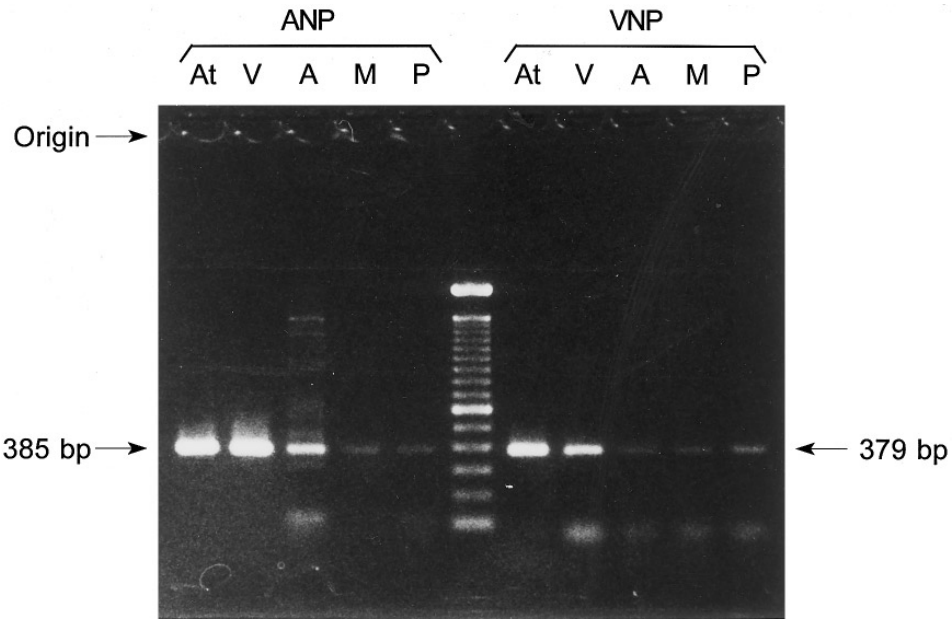

FIG. 4. UV image of ethidium bromide-stained RT-PCR products after 2% agarose gel electrophoresis. Lanes 1-5 contain ANP RT-PCR product (385 bp) from atrium (At), ventricle (V), anterior intestine (A), middle intestine (M) and posterior intestine (P). Lane 6 contains a 100-bp ladder. Lanes 7-11 contain VNP RT-PCR product (379 bp) from atrium (At), ventricle (V), anterior intestine (A), middle intestine (M), and posterior intestine (P).

through their sensitivity to secretogogues (nutrients such as glucose and amino acids) in the luminal compartment. Preliminary data from our laboratories suggest enhanced NP release from eel intestine in response to both physical and chemical stimuli (personal observation). The local synthesis and release, and paracrine delivery of NP would afford flexible control over transport either directly through inhibitory action on enterocytes or indirectly through neuromodulation of enteric neurons. Additionally, NP may have secondary, indirect effects on ion transport *in vivo* through changes in motility or local blood flow via effects on intestinal and vascular smooth muscle, respectively (1, 21, 25).

A speculative model has been proposed for the local role of NP in regulating teleost intestinal absorption (3). The premise for this model is the competition between coupled Na^+-Cl^- and Na^+-nutrient cotransport systems in the apical (lumen-facing) membrane for the finite Na^+ electrochemical gradient. The local inhibition by NP of Na^+-Cl^- cotransport in the presence of digesta could reduce this competition to promote Na^+-nutrient uptake in that segment without reducing in other intestinal segments the salt transport necessary for iono- and osmoregulation.

In support of a model for local regulation, the biochemical studies reported herein document the production of two natriuretic peptides (ANP and VNP) in the intestine of the Japanese eel. In HPLC analysis, peaks in immunoreactivity occurred at the empirically-determined elution times of authentic eel NPs, thereby confirming identity as eANP and eVNP. The broad peaks with shoulders in HPLC profiles may represent additional truncated forms of NP due to enzymatic activity in the intestine. Using RT-PCR analysis, ANP and VNP mRNA expression was detected in all three intestinal segments. The ANP RT-PCR product was especially abundant in the anterior intestine, where nutrient absorption is expected to be greatest, compared with more posterior intestinal segments. Consistent with the biochemical findings, both ANP and VNP are effective inhibitors intestinal salt transport in fishes, including the eel (2, 3).

The immunohistochemical findings place NP production in cells of the epithelium, where the luminal contents can be chemically sensed and, consistent with the model, a physiologically-appropriate local secretory response generated. The band of subepithelial immunoreactivity also suggests targets in the underlying muscular layers where there may occur changes in peristalsis and vascular flow relevant to the transit of the digesta and the removal of absorbed nutrients.

The intestinal mucosa contains much less NP than the eel heart. Without correction for chromatographic recovery, mucosal eANP and eVNP contents calculated here on a fmol/mg tissue basis are only a few percent of the corresponding mass-specific NP contents of eel heart (20). This is reflective of the lower relative abundance of NP-containing cells in the intestine compared with the heart determined immunohistochemically, and of the lesser abundance of RT-PCR product for

intestinal tissues compared with that for heart. The observation that NP contents and immunohistochemistry differ little in the mucosa of FW and SW eels is consistent with a role for NP in the regulation of salt and nutrient absorption, which would be necessary independent of environmental salinity. Major salinity-dependent alterations in hormone production, storage, and local release cannot be ruled out, however, since the data gathered here provide only a snapshot view of hormone content with little information on the dynamic operation of the system. Nevertheless, the data presented here provide the biochemical and immunohistochemical support for a paracrine regulatory scheme.

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