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ATRIAL NATRIURETIC PEPTIDE GENE EXPRESSION IN THE RAT GASTROINTESTINAL TRACT

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SUMMARY: The presence of ANP prohormone immunoreactivity in rat GI tract suggests that it may be an extracardiac site of ANP synthesis. The aim of this study was to investigate the expression of ANP mRNA in the adult rat GI tract. ANP mRNA was detected by ribonuclease protection analysis in stomach, small and large intestines, and rectum/anus. The highest concentrations of ANP transcripts were found in the proximal stomach, antrum, proximal colon, and rectum/anus at levels that ranged from 1 to 10% of that found in cardiac ventricle. Northern blot analysis of total RNA from these tissues identified a single 0.9 kb ANP transcript similar to that detected in heart. Gel filtration chromatography of tissue extracts provided evidence for the presence of the complete ANP prohormone in proximal stomach, antrum, proximal colon and rectum/anus. These results demonstrate that the gene for ANP is expressed in specific regions of the rat GI tract,suggesting that tissue-specific differential regulation of ANP synthesis occurs within the GI tract. © 1994 Academic Press, Inc.

Atrial natriuretic peptide (ANP), synthesized and stored primarily in atrial myocytes, is a peptide hormone with potent natriuretic, diuretic, and vasoactive effects (1,2). Although the cardiac atria represent the major sites of ANP synthesis, evidence for the expression of the ANP gene has been demonstrated in a large number of extraatrial tissues including the colon (3). ANP immunoreactivity has been found in extracts of rat stomach, small and large intestines (4,5,6), guinea pig jejunum and colon (7), human stomach, duodenum, jejunum, colon, and rectum (8,9). Chromatographic analysis of tissue extracts from colon and small intestine demonstrated the presence of both mature ANP (amino acid residues 99-126 of the prohormone) and the complete prohormone or proANP(1-126) (4,5,8). Since proANP(1-126) is not found in the circulation (10) and is found only within intracellular compartments, these data suggest that endogenous ANP may be synthesized in the intestine.

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Numerous reports have indicated that the intestine is a target organ for ANP, demonstrating various biological activities (11,12,13,14), as well as specific binding sites for ANP (15,16,17,18), raising the possibility that ANP may act as a local mediator or paracrine effector of gastrointestinal (GI) function. To examine whether or not ANP is produced *de novo* in the rat GI tract and to determine if there may be regional differences in the expression of the ANP gene, we have determined the sites of expression of the ANP gene in rat stomach and intestine using ribonuclease protection analysis. In those regions of the GI tract where ANP mRNA was relatively abundant we assayed for immunoreactive proANP(1-126).

MATERIALS AND METHODS

Peptide extraction: Heart (atria, ventricle), stomach (proximal, fundus, antrum), small intestine (duodenum, jejunum, ileum), large intestine (cecum, proximal, mid, and distal colon), rectum and anus tissues *in toto* were removed from fasted adult male Sprague-Dawley rats (300-400g) following decapitation, boiled for 10 minutes in 10 volumes of 1 N acetic acid containing 0.1 mM phenylmethylsulfonyl fluoride, homogenized with a polytron homogenizer for 60 seconds. The homogenate was centrifuged at 27,000xg for 15 minutes at 4°C, and the supernatant (crude extract) was stored at -80°C. Some crude extracts were applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA) equilibrated with 0.1 N acetic acid and eluted with a mixture of acetonitrile and 0.1 M trifluoroacetic acid (60:40). The eluate was dryed under nitrogen, resuspended in a minimal volume of radioimmunoassay (RIA) buffer, assayed for immunoreactive ANP and proANP(1-30), then subjected to high performance gel permeation chromatography. The recoveries of added rat ANP and proANP(1-30) were 78.2 ± 0.3% (mean ± SEM; n = 3) and 65.0 ± 0.7%, respectively.

Measurement of ANP and proANP(1-30): Estimation of immunoreacitve rat ANP and proANP(1-30) in crude extracts of heart and GI tissues, Sep-Pak C_{18} effluents, and column fractions was performed by RIA as described in detail elsewhere (19,20). The antibody used in the ANP RIA recognizes ANP as well as the carboxy-terminus of the complete prohormone and does not cross-react with residues from the amino terminus of the prohormone. The antibody used in the proANP(1-30) RIA recognizes the thirty amino acid peptide proANP(1-30) derived from the amino terminus of the prohormone as well as the complete prohormone and exhibits no cross-reactivity with the carboxy-terminus of the prohormone or ANP (10).

High performance gel permeation chromatography (HP-GPC): HP-GPC was performed on a TSK-Gel G2000SW column (7.5 x 600 mm) equipped with a guard column (7.5 x 75 mm) (TosoHaas, Montgomeryville, PA) as described previously (21). The column was calibrated with blue dextran (Vo), *p*-aminohippuric acid (Vt), myoglobin (16.9 kD), cytochrome c (12.4 kD), vasoactive intestinal peptide (3.3 kD), (Tyr^1)-somatostatin (1.7 kD), synthetic rat ANP, and synthetic human proANP(1-30).

RNA extraction and ribonuclease protection analysis: Freshly harvested tissues (100 mg) were homogenized and total RNA extracted with RNAzol B (TEL-TEST, Inc., Friendswood, TX). Total RNA was hybridized with an antisense [^{32}P]UTP labeled RNA probe prepared by *in vitro* transcription of a *Eco*RI/*Xho*I linearized cDNA template for rat ANP. The original full-length rat ANP cDNA in the pUC-9 plasmid was a generous gift from Dr. David Gardner (University of California-San Francisco) (22). The *Eco*RI/*Hind*III fragment was isolated and subcloned into pGEM4z (Promega Corp., Madison, WI) with the orientation of T7 promoter giving cRNA and SP6 promoter yielding the mRNA. The cRNA probe (278 nucleotides) spans exon 3 of the rat ANP gene and protects 251 nucleotides of the ANP mRNA (see Fig. 2A). An actin probe (132 nucleotide protected fragment) synthesized from a template containing a 249 bp fragment of the β-actin gene (Ambion, Inc., Austin, TX) was included in the hybridization reactions as an internal control. Total RNA (5-40 μg) and gel-purified probes for rat ANP (2.5-3.5 x 10^4 cpm) and β-actin (1.0 x 10^4 cpm) were precipitated with ethanol, resuspended in hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 0.4 M sodium acetate), denatured at 90°C for 5 minutes, and annealed overnight at 45°C. Ribonuclease digestion buffer containing 6 units of RNase One (Promega) was added and incubated

for 60 minutes at 37°C. SDS (0.15%) was added to the mixture to stop digestion, then tRNA (1 μg/ml) and ethanol were added to precipitate the RNA. The precipitate was washed with ethanol, dried, resuspended in gel loading buffer (80% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% SDS), then heated at 70°C for 10 minutes. RNA was fractionated on a 6% denaturing polyacrylamide gel, and analyzed by autoradiography. The hybridization signal was quantified by 2-D scanning. The bit map was analyzed by Scan Analysis software (BioSoft, Ferguson, MO). Signals obtained with the ANP probe were normalized to those obtained for the actin probe. Sensitivity of the protection assay was determined by assay of sense RNA prepared from the rat ANP cDNA using SP6 polymerase. The least amount of specific mRNA that could be detected by the ANP probe was 1 pg following 18 hours of exposure to Kodak X-OMAT AR film.

Northern blot analysis: Total RNA (10-20 μg) was fractionated by electrophoresis on formaldehyde-1.6% agarose gels and transferred to nylon membranes (Zeta-Probe, Bio-Rad, Hercules, CA). Hybridizations were performed using the full-length rat ANP cDNA probe (~800-bp *Eco*RI/*Hind*III fragment) labeled by the random priming method with [^{32}P]dCTP as previously described (23). To control for RNA loading and to determine efficiency of transfer, membranes were stained with ethidium bromide to visualize 18S and 28S rRNA.

RESULTS

Initial attempts to detect ANP mRNA in total RNA extracts of rat GI tissues by Northern blot analysis gave inconsistent results. Although a strong hybridization signal of 0.9 kb in size was always detected in total RNA extracts from cardiac atria and ventricle, only rarely was a weak hybridization signal detected in total RNA from GI tissues. As depicted in Figure 1, occasionally an ANP transcript similar in size to that expressed in cardiac tissues could be detected in proximal stomach, antrum, and rectum/anus, but not from fundus, duodenum, jejunum, ileum, colon or cecum. Attempts to increase sensitivity by increasing the amount of total RNA to 40 μg and substituting 5 μg of poly[A]$^+$ RNA for total RNA failed to produce a significant improvement in assay results.

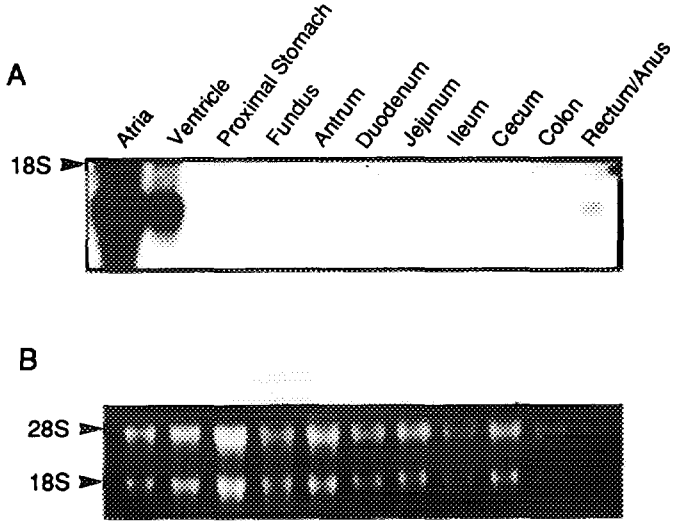

Figure 1. Northern blot analysis of ANP gene expression in cardiac and designated GI tissues of the adult rat. Total RNA from atria (10 μg), ventricle (20 μg), and designated regions of GI tract (20 μg) was fractionated and transferred to a nylon membrane as described in Materials and Methods. (**A**) Membrane probed with [^{32}P]-labeled rat ANP cDNA; (**B**) same membrane was stained with ethidium bromide.

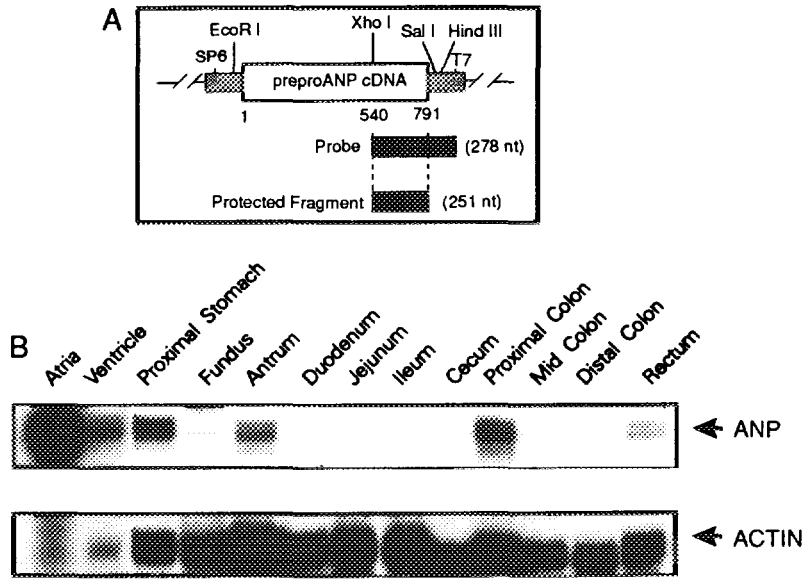

Figure 2. ANP expression in adult rat heart and GI tract. (**A**) Schematic representation defining the probe and protected fragment utilized in the analysis. (**B**) Representative ribonuclease protection analysis of total RNA from atria (5 μg), ventricle (10 μg), and designated regions of the gastrointestinal tract (40 μg). Positions of protected fragments for ANP and β-actin are shown.

Subsequently, to enhance the sensitivity of our analysis we devised a ribonuclease protection assay utilizing a probe specific for the 3′ terminus of the ANP mRNA (Fig. 2A). A representative analysis is shown in Figure 2B. Routinely, ANP transcripts were detected not only in atria and ventricle but also in proximal stomach, antrum, proximal colon, and rectum/anus. Quantification of the ANP mRNA levels in these GI tissues (normalized to actin mRNA levels) from three adult rats are shown in Figure 3. These results confirmed the modest level of expression of the ANP gene in

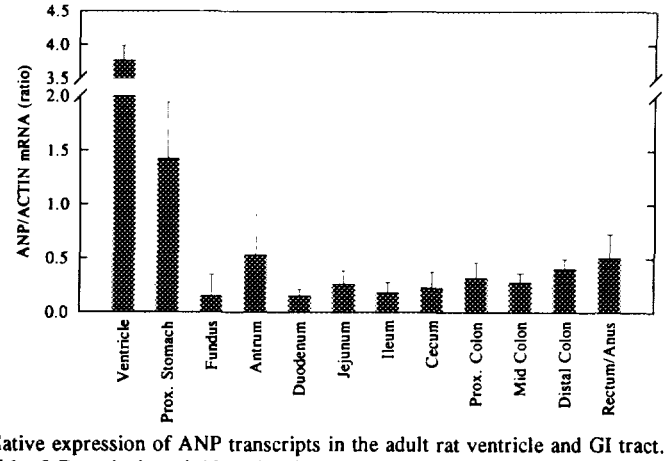

Figure 3. Relative expression of ANP transcripts in the adult rat ventricle and GI tract. Quantitation was performed by 2-D analysis and ANP signals were normalized to β-actin. Data is average $\pm$ SEM (n=3).

the GI tract that we first observed from Northern blot analysis. The relative level of ANP mRNA in these tissues compared to ventricle averaged from a low of 1% (duodenum) to a high of 10% (proximal stomach). ANP expression was consistently found to peak in proximal stomach, antrum, colon, and rectum/anus. The level of ANP transcripts decreases dramatically in fundus to reach low to undetectable levels in duodenum, jejunum, and ileum.

To determine whether the mRNA for ANP is translated into the ANP prohormone within the rat GI tract, boiling acid extracts of proximal stomach, fundus, antrum, proximal colon, and rectum/anus were fractionated on HP-GPC and fractions were assayed for immunoreactive ANP and proANP(1-30). In atria, both immunoreactivites co-eluted forming a single major peak with an apparent molecular weight of 12,500 kD. These results are in agreement with the findings of others demonstrating that the major storage form of ANP in rat atria is the complete prohormone or proANP(1-126)(24). A similar peak of co-eluting ANP and proANP(1-30) immunoreactivities was observed in proximal stomach, antrum, proximal colon and rectum/anus. However, in contrast to atria, this peak represented only a portion of both immunoreactivites. Multiple peaks of smaller ANP immunoreacitivy were observed in all tissues with the major peak eluting at a position similar to synthetic rat ANP. Peaks of immunoreactive proANP(1-30) lower in apparent molecular weight than proANP(1-126) were observed in all tissue extracts, some of which co-eluted with synthetic proANP(1-30). In contrast, protein extracts of fundus contained mainly the low molecular weight species of immunoreactivity and exhibited only a very small amount of the high molecular weight ANP and proANP(1-30) immunoreactivities. (Fig. 4).

DISCUSSION

In this study, we have detected the presence of ANP mRNA in the rat GI tract utilizing ribonuclease protection analysis. Specifically, we found relatively high levels of ANP mRNA in the proximal (nonglandular) and antropyloric regions of the stomach, colon, and rectum/anus. The abundance of ANP transcripts in these tissues relative to that found in the heart is comparable to the ANP mRNA concentrations reported in lung and pituitary (2). Additional evidence that the ANP mRNA in GI tract is similar to that found in the heart comes from the results of the Northern blot analysis which shows that the size of the GI ANP transcript was similar to that found in cardiac tissues. With the exception of colon, this is the first report to demonstrate the presence of ANP mRNA in GI tissues of any species. The differential expression of a peptide hormone gene is a characteristic commonly associated with regulatory peptides synthesized in the GI tract (25) and suggests region-specific regulation of the synthesis and secretion of ANP in the GI tract. Whether this regional regulation of ANP gene expression reflects functional differences for ANP remains to be determined.

Evidence that the ANP mRNA is translated *in vivo* in these specific regions comes from the detection of prohormone-like immunoreactivity in extracts of these tissues. These results are consistent with previous findings from our laboratory as well as those of others that have

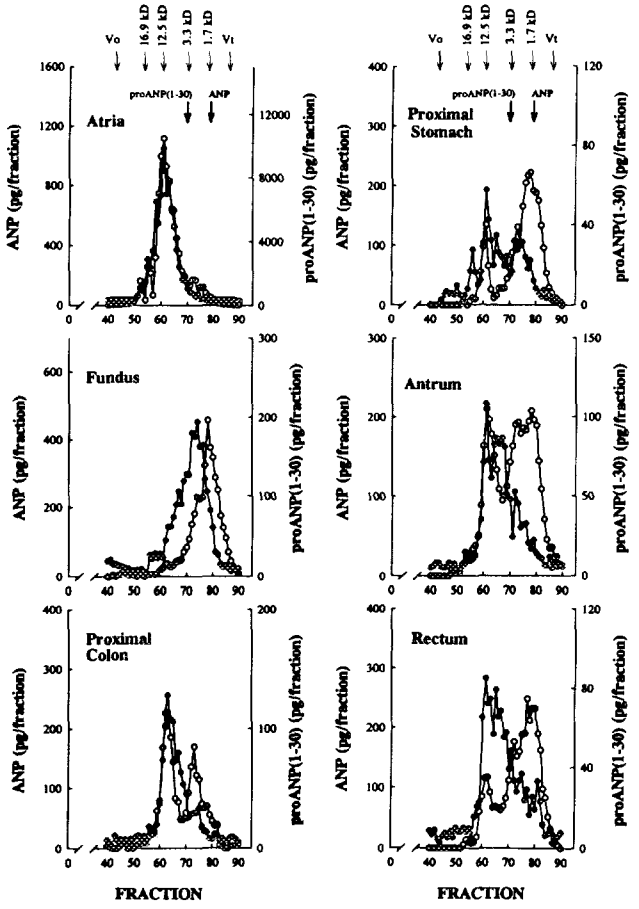

Figure 4. Gel filtration profiles of ANP and proANP(1-30) immunoreactivity in extracts of adult rat cardiac and designated GI tissues. Arrows indicate the elution positions of protein molecular weight calibrators, synthetic human proANP(1-30), rat ANP, void volume (Vo), and total volume (Vt).

demonstrated the presence of immunoreactive prohormone in extracts of rat stomach and colon (5,6). Vuolteenaho, et al. (4) examined acid extracts of whole rat stomach by gel filtration chromatography coupled with ANP RIA and found multiple species of immunoreactivity including proANP(1-126) and ANP. Our studies agree with these findings and, in addition, demonstrate that this complex population of ANP molecular species is localized primarily to the proximal and distal regions of the stomach but is absent in the fundus. Since we found appreciable amounts of ANP mRNA in proximal stomach and antrum, but very little in fundus, it is likely that post-translational processing of the prohormone synthesized in proximal stomach and antrum are responsible for these different patterns of ANP molecular species. Indeed, this heterogenous pattern of immunoreactive forms has been found in other extraatrial tissues that synthesize ANP, e.g. brain, lung, adrenal gland, placenta (2). Whether this reflects tissue-specific differences in intracellular processing of the prohormone and/or extraction of circulating ANP molecular forms will require further study. The lack of appreciable amounts of proANP(1-126) immunoreactivity in the fundus and the inability of our assays to detect a

significant amount of ANP mRNA in this region of the stomach argues strongly that the ANP gene is not expressed in this tissue. Further studies will be required to determine the mechanism responsible for this complex pattern of immunoreactive ANP species in gastric tissues.

Taken together, these studies provide definitive evidence for the *de novo* synthesis of ANP within the GI tract of the rat. Previous evidence from immunohistochemical studies of pig and human colon have demonstrated the presence of ANP prohormone immunoreactivity in lymphoid and enterochromaffin cells, respectively (7,9). Since whole tissue extracts were used in the present study, it is unclear precisely where ANP is synthesized in these rat GI tissues. Studies are in progress to localize ANP mRNA expression using *in situ* hybridization.

Analogous to other extraatrial sites of ANP synthesis, the GI tract displays considerably less steady-state ANP mRNA than cardiac tissues. It is unlikely that GI ANP contributes substantially to circulating ANP, but it raises the possibility that GI ANP may act locally through neural, endocrine or paracrine pathways to modulate GI function. Several reports have demonstrated that atrial natriuretic peptides have potent biological effects within GI tissues. In the rat, ANP has been shown to produce contractions of the duodenum (12), induce relaxation of the longitudinal layer and contraction of the circular layer of small intestinal smooth muscle *in vitro* (26), suppress Na^+-coupled glucose transport in the small intestine (13), and increase intestinal Na^+ and water adsorption (14). Furthermore, specific ANP receptors coupled to particulate guanylate cyclase have been identified in the rat small intestine and colon (15,17,27) That ANP may function as a neurotransmitter in the GI tract is supported by the knowledge that ANP has been located in the peripheral nervous system (28,29) and that when administered intracerebroventricularly, ANP stimulated acid secretion in the anesthetized rat (30). Alternatively, as hypothalamic ANP has recently been demonstrated to modulate the hormonal release of other regulatory peptides, e.g. prolactin (31), vasopressin (32), and ACTH (33) from the pituitary, the possibility that GI ANP may function in a similar fashion regulating the release of various GI regulatory peptides from GI tissues must be considered. Evidence for VIP mediating the effect of ANP on Cl^- secretion in rat proximal and distal colon has been reported (17,34).

The expression of ANP in gastric tissues coupled with the evidence that ANP receptors have been identified in pig chief cells suggests a possible paracrine function for this peptide in stomach (35). The previously mentioned report describing the vagal-dependent stimulation of gastric acid secretion by ANP (30) suggests a neural role for ANP in rat stomach. However, [^{125}I]ANP autoradiographic visualization studies of rat stomach have revealed little or no binding sites in gastric tissues (15,16,17). It is possible that ANP receptors are present in rat stomach but in low abundance and have previously gone undetected in gastric tissues. In light of its pronounced effect on relaxation of vascular smooth muscle (2), ANP may play a role in gastric mucosa vasodilation (36). Our finding that there may be a link between the secretion of gastrin and that of ANP (5), coupled with the present finding of ANP expression in the same vicinity as that for gastrin, suggests that antral ANP might mediate gastrin-induced gastric mucosal vasodilation. Previous studies have

demonstrated that pentagastrin-induced increase in gastric mucosal blood flow is independent of acid secretion. Evidence suggests that gastrin's effect is produced directly by a vasodilator activity, either gastrin itself or through the action of an alternative vasoactive agent induced by gastrin (26). Recent findings have ruled out the possible role of histamine as the mediator of gastrin's effect (37). It is tempting to speculate that since both adrenal chromaffin cells and enterochromaffin cells in the colon have been shown to synthsize ANP (38,9), and since enterochromaffin cells are known to occur in the antrum (24), and have neurosecretory granules similar in appearance to those described in cardiocytes of the atria (39), it is possible that these may be the cells which synthsize ANP in the gastric antrum.

The mammalian GI tract synthesizes and secretes several regulatory peptides with a variety of effects on GI function (40). The present findings coupled with the previous demonstration of ANP immunoreactivity and ANP receptors within the intestine and stomach provide strong evidence for a possible role for this peptide in the regulation of GI physiology. Additional studies are needed to elucidate the precise physiological and/or pathophysiological role(s) of GI ANP.

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