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Presence of Atrial Natriuretic Factor Prohormone in Enterochromaffin Cells of the Human Large Intestine

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Atrial natriuretic factor is a hormone intimately involved in water and salt homeostasis. The heart constitutes the major but not exclusive site of synthesis of this hormone. Among other functions, the gastrointestinal tract has endocrine functions, plays an important role in volume regulation of the body, and seems to be a target organ for atrial natriuretic factor. Therefore, the presence of atrial natriuretic factor was investigated in the human gut. Immunoreactive atrial natriuretic factor was found in intraoperatively obtained samples of normal human colon. Acidic extracts of human large intestine contained about 0.4 pmol/g wet wt of atrial natriuretic factor. Analysis of atrial natriuretic factor immunoreactivity by gel-filtration and reverse-phase high-performance liquid chromatography showed that about 65% of the immunoreactivity corresponded to the atrial natriuretic factor prohormone and about 35% corresponded to the C-terminal $ANF_{99\text{-}126}$. Immunohistochemistry showed atrial natriuretic factor prohormone location in enterochromaffin cells of the colon mucosa. Altogether, these findings show the presence of atrial natriuretic factor prohormone in enterochromaffin cells of the human large intestine and may suggest this organ as a site of atrial natriuretic factor synthesis in humans.

Atrial natriuretic factor (ANF) is involved in salt and water homeostasis. It was first discovered in mammalian atrial myocytes, where it is synthesized and stored as a 126-amino-acid prohormone (1,2). Upon appropriate stimulation, the prohormone is cleaved and released into the circulation where the C-terminal 28-amino-acid peptide $ANF_{99\text{-}126}$ has been identified as a biologically active hormone (3,4). Subsequently, the presence of ANF has been determined in several tissues other than the heart (5–9), and recently it has been discovered in guinea pig and rat intestine (10,11). The gastrointestinal tract plays an important role in volume regulation. Moreover, it seems to be a target organ for ANF with specific ANF binding sites (12–17). These findings, together with the endocrine properties of the gut (18–21), a major organ under endocrine control, might suggest a role of ANF in the intestine. Therefore, the aim of this study was to investigate the presence of ANF in the intestines of human subjects.

Materials and Methods

Tissues

Large intestinal tissue was obtained from 14 human subjects during surgery for the resection of tumors of the colon. Segments of nontumorous tissue (1–2 g) were washed with 4°C saline immediately after resection and were added to 4 mL of 0.1N HCl for extraction or were fixed in Bouin's fluid for immunohistochemistry.

Extraction Procedure

Tissue sections were boiled in 0.1N HCl in a microwave oven for 5 minutes and were subsequently homogenized with a polytron for 30 seconds. The resulting homogenate was boiled for another 5 minutes and centrifuged subsequently for 30 minutes at 4°C (20,000 × *g*). The supernatant was applied to small columns filled with

Abbreviations used in this paper: **ANF, atrial natriuretic factor; proANF, atrial natriuretic factor prohormone; TFA, trifluoroacetic acid.**

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amberlite XAD-2 adsorbent resin (Serva, Heidelberg, Germany). The columns were washed with 0.1% trifluoroacetic acid (TFA) and eluted with 4 mL of 80% acetonitrile in 0.1% TFA. The eluates were lyophilized for further analysis.

Radioimmunoassay and Recovery Determination

The radioimmunoassay (RIA) was performed as described previously (22,23); antibody Toni III (gift of R. Arendt) directed against the carboxy terminus of ANF recognizes $ANF_{99\text{-}126}$ as well as the ANF prohormone (proANF). Recovery of immunoreactive ANF was evaluated by adding 250 or 500 fmol of $ANF_{99\text{-}126}$ or $proANF_{2\text{-}126}$ (gifts of Drs. Schöne, Preibisch, and Seipke, Höchst AG, Frankfurt, Germany) to the acidic tissue homogenate and performing extraction and separation procedures.

Chromatographic Analysis of Atrial Natriuretic Factor Immunoreactivity

Gel filtration. The lyophilized extract of colonic tissue was dissolved in 250 μL of 0.1% TFA and loaded on a Sephadex G 50 column (860 × 16 mm; Pharmacia, Uppsala, Sweden). The dynamic phase was 0.1N acetic acid, the flow rate was 10 mL/h, and the fraction size was 2.5 mL. Calibration was performed with albumin (V_o), vitamin B_{12} (V_t), $proANF_{2\text{-}126}$, and $ANF_{99\text{-}126}$.

Reverse-phase high-performance liquid chromatography. An aliquot of the pooled lyophilized fractions obtained by gel filtration was taken up in 50 μL 0.5% TFA and loaded on a high-performance liquid chromatography (HPLC) C_8 column (5 μm, 750 × 4.6 mm; Beckman Instruments, San Ramon, CA). Elution was performed with a linear gradient of acetonitrile (20%–55%, 55 minutes) in 0.1% TFA (flow rate, 0.5 mL/min). Calibration of the column was carried out with $ANF_{99\text{-}126}$ and $proANF_{2\text{-}126}$.

Immunohistochemistry

Colonic tissue specimens fixed in Bouin's fluid were dehydrated in alcohols and embedded in paraffin. An indirect immunoperoxidase method was carried out as previously described (25). In brief, 5-μm sections of the Bouin-fixed, paraffin-embedded tissues were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was quenched by sequential treatment with 7.5% hydrogen peroxidase, 2.28% periodic acid, and 0.02% sodium borohydride. After overlaying the tissue in 2% normal goat serum, the specific antibodies were applied as follows: antibody Toni III, reacting with both proANF and $ANF_{99\text{-}126}$, was diluted in phosphate-buffered saline (PBS) at a ratio of 1:400; antibody GT-23 (24), raised against the N-terminal fragment of proANF and reacting with the prohormone but not with $ANF_{99\text{-}126}$, was used in a 1:100 dilution; and the mouse monoclonal antibody raised against human chromogranin [LK 2 H 10; (26)] was diluted 1:200. Incubation time was 2 hours at 37°C for each antibody. After washing in PBS, peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) G or goat anti-mouse IgG, diluted 1:250 and 1:1800, respectively, was applied for 5 minutes at 22°C. Peroxidase reaction was visualized with aminoethylcarbazole, and hematoxylin was used to counterstain the nuclei. Controls included substitution of first and/or second antibody by PBS, normal mouse serum, or normal rabbit serum.

Materials

Human $ANF_{99\text{-}126}$ was purchased from Nova Biochem, Läufelfingen, Switzerland; rat $proANF_{2\text{-}126}$ was a gift from Drs. Schöne, Preibisch, and Seipke, Höchst AG, Frankfurt, Germany. ^{125}I-iodinated $ANF_{99\text{-}126}$ was obtained from Biotrend, Cologne, Germany (sp act, 2000 Ci/mmol). Antibody Toni III was a gift from R. Arendt, Medizinische Klinik I, Klinikum Großhadern, University of Munich, Munich, Germany; antibody GT-23 was provided by Drs. M. Cantin and G. Thibault, Clinical Research Institute, Montreal, Quebec, Canada. Amberlite XAD resin and sodium borohydride were obtained from Serva, Heidelberg, Germany. Normal goat, rabbit, and mouse sera and peroxidase goat anti-mouse IgG were obtained from Nordic Biogenzia, Bochum, Germany. Hydrogen peroxide and periodic acid were obtained from Merck, Darmstadt, Germany. Peroxidase goat anti-rabbit IgG, aminoethylcarbazole, and all other chemicals were obtained from Sigma, Taufkirchen, Germany.

Results

Large intestine tissue samples preextracted by XAD resin and purified by Sephadex G 50 gel filtration contained 0.4 pmol/g wet wt ANF immunoreactivity. Recovery rates of $ANF_{99\text{-}126}$ and $proANF_{2\text{-}126}$ were 27% and 21%, respectively. After Sephadex G 50 gel filtration of the tissue extracts, the majority of ANF immunoreactivity coeluted with the prohormone (Figure 1*A*). When the ANF fractions obtained by gel filtration were subjected to reverse-phase HPLC, about 65% of the immunoreactivity coeluted with the prohormone and 35% with $ANF_{99\text{-}126}$ (Figure 1*B*).

The localization of ANF immunoreactivity was analyzed by immunohistochemistry. The antibody Toni III, directed against both the prohormone and the $ANF_{99\text{-}126}$ segment, showed specific intracytoplasmic staining of single epithelial cells within the colon mucosa (Figure 2*A*). On parallel sections, virtually identical staining patterns were achieved using antibody GT-23 (Figure 2*B*) and antibody against chromogranin (Figure 2*C*). No immunostaining was seen at other locations, particularly not in stromal or plasma cells. Controls were always negative.

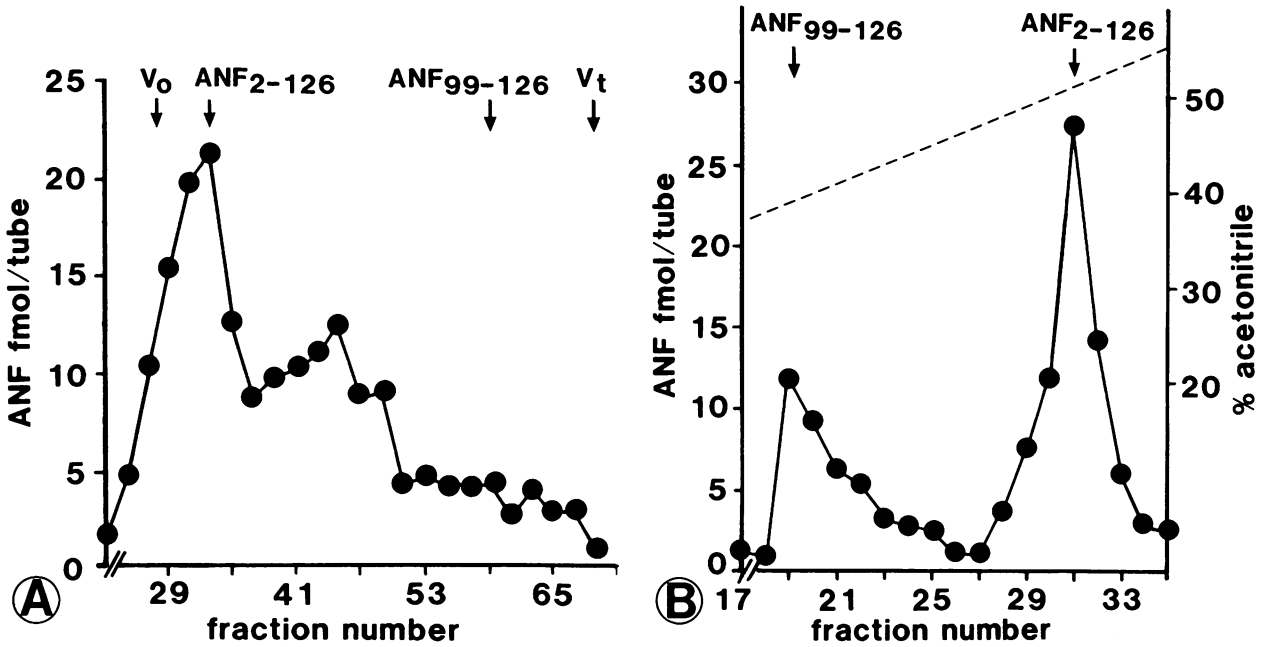

Figure 1. Characterization of ANF immunoreactivity.

A. Separation of ANF immunoreactivity in extracted colonic tissue samples by Sephadex G 50 gel filtration. Calibration was performed with albumin (V_o), vitamin B_{12} (V_t), $proANF_{2\text{-}126}$, and $ANF_{99\text{-}126}$. The majority of ANF immunoreactivity elutes in the range of $proANF_{2\text{-}126}$.

B. Characterization of the ANF immunoreactivity obtained by reverse-phase HPLC. About 65% of the immunoreactivity coelutes with $proANF_{2\text{-}126}$, whereas about 35% coelutes with the C-terminal $ANF_{99\text{-}126}$.

Discussion

Initially the cardiac atria were considered to be the major source of ANF synthesis. However, subsequent reports found evidence for release of ANF in organs other than the heart (5,6), and recently the presence of ANF has been shown in the intestine of the guinea pig and the rat (10,11). Because the intestinal tract plays an important role in fluid regulation of the body, this finding, together with the demonstration of ANF binding sites in the gut (12,14,17), suggested a possible role of this hormone in mammalian intestinal fluid absorption. Indeed, in various species, ANF has been shown to influence intestinal fluid and electrolyte handling (13,15,16,27), whereas in others there was no effect (28,29).

In the present study we found evidence for the presence of ANF prohormone in enterochromaffin cells of the human large intestine. This assumption is based on the following findings:

1. Atrial natriuretic factor immunoreactivity could be determined in extracted and purified colonic tissue samples of human subjects.
2. High-performance liquid chromatography analysis showed elution of the majority of the ANF immunoreactivity with the ANF prohormone. About one third of the immunoreactivity coeluting with $ANF_{99\text{-}126}$ might be due to degradation of the prohormone, which occurs rapidly in the gut (10).
3. Immunohistochemical staining with two different antibodies showed the presence of ANF prohormone in cells of the large intestine. The same cell type was stained with the antibody against chromogranin, highly suggesting that ANF immunoreactivity and most likely ANF synthesis is localized in enterochromaffin cells.

Enterochromaffin cells contribute to the endocrine regulation of the gut by containing and releasing various hormones (30,31), and the biosynthesis and secretion of ANF has been shown in chromaffin cells of the adrenal glands (32).

In the present study we examined colonic tissue, but we have also found ANF immunoreactivity in tissue extracts of the jejunum and the ileum (data not shown). Recently, immunohistochemical evidence for ANF was found in biopsy samples of various parts of the gastrointestinal tract, but characterization of the ANF immunoreactivity or the ANF-containing cells was not performed (33). Thus, the regional distribution, cellular localization, and characterization of ANF immunoreactivity in other parts of the human intestine remains to be elucidated.

Altogether, our results for the first time suggest that

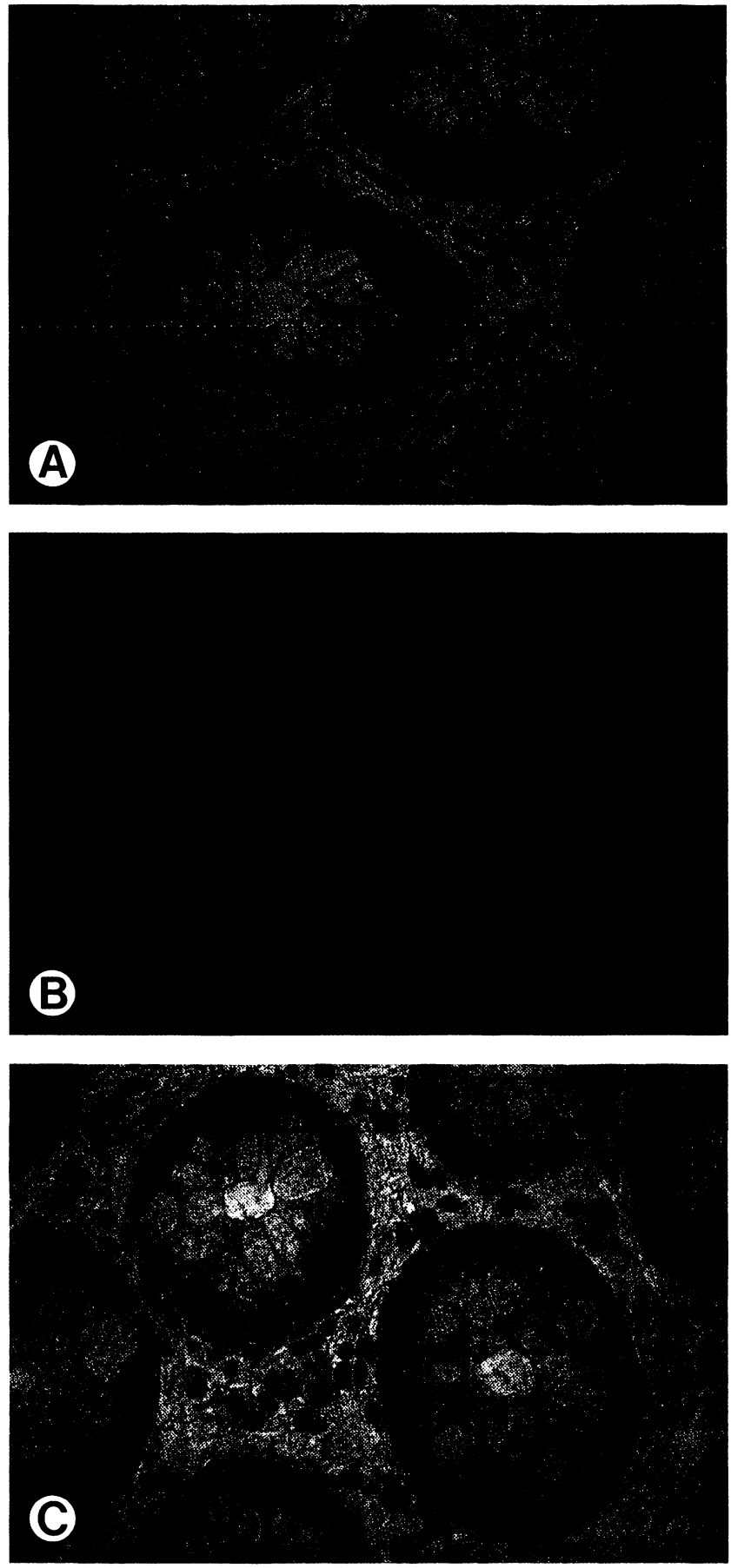

Figure 2. Immunohistochemistry in a sigmoid colonic tissue sample. All three figures show 5-μm parallel paraffin sections of a Bouin-fixed sigmoid colon tissue block with indirect immunoperoxidase reactions stained with aminoethylcarbazole and with nuclear hematoxylin counterstaining (original magnification ×560).

A. Antibody Toni III, which recognizes both proANF and $ANF_{99\text{-}126}$, shows strong specific immunostaining of cytoplasm of single cells within the mucosa epithelium.

B. Antibody GT-23, which reacts with proANF but not with $ANF_{99\text{-}126}$, shows the same pattern and distribution of specific immunostaining as shown in *A*.

C. The antibody against chromogranin shows the specifically stained enterochromaffin cells with a virtually identical staining and distribution pattern as shown in *A* and *B*, suggesting that proANF is present in enterochromaffin cells of the human colon.

the large intestine is a site of ANF synthesis in humans. The physiological and pathophysiological implications of this finding could be of considerable interest.

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