Cellular Mechanisms for Synthesis and Secretion of Atrial Natriuretic Peptide and Brain Natriuretic Peptide in Cultured Rat Atrial Cells


To investigate the cellular mechanism for the synthesis and secretion of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), we examined the effects of vasoactive agents on the secretion rates and gene expression of ANP and BNP in cultured rat atrial cells. Endothelin (10⁻⁷ M, +61%), 12-O-tetradecanoylphorbol 13-acetate (TPA, 10⁻⁴ M, +62%), the calcium ionophore A23187 (10⁻⁶ M, +95%), and Bay K 8644 (10⁻⁶ M, +34%) (p < 0.05 each) all increased the secretion of ANP into the culture media in a dose-dependent fashion. On the other hand, endothelin (10⁻⁷ M, +57%) and TPA (10⁻⁴ M, +55%) (p < 0.01 each) increased the secretion of BNP in a dose-dependent manner, whereas A23187 (10⁻⁶ M, −45%, p < 0.001) suppressed the secretion of BNP in a dose-dependent manner, and Bay K 8644 caused no significant effects on BNP secretion. The molecular forms of intracellular ANP were exclusively γ-ANP, whereas those of BNP were γ-BNP and its carboxy terminal 45-amino-acid peptide, BNP-45. The ratio of media to cell contents was much higher in BNP than in ANP. Northern blot analysis revealed that both ANP mRNA and BNP mRNA levels were significantly increased by 10⁻⁷ M endothelin (ANP mRNA, +52%; BNP mRNA, +36%; p < 0.05 each) and 5×10⁻⁴ M 1-oleoyl-2-acetylglycerol (ANP mRNA, +296%; BNP mRNA, +133%; p < 0.01 each) but not by 10⁻⁶ M A23187. Thus, the secretion of ANP is stimulated by both the elevation of [Ca²⁺], and the activation of protein kinase C, whereas its synthesis is increased mainly by the activation of protein kinase C. The synthesis and secretion of BNP are augmented by the activation of protein kinase C rather than the elevation of [Ca²⁺]. Furthermore, the processing and secretion of ANP and BNP may be regulated in different manners. (Circulation Research 1992;71:1039–1048)

KEY WORDS • calcium • protein kinase C • endothelin • mRNA

Atrial natriuretic peptide (ANP) is a peptide hormone, a major part of which is stored in and secreted from cardiac atrial cells, whose amino acid sequences are highly conserved from rodents to mammals. Although brain natriuretic peptide (BNP), originally isolated from porcine brain,1 is also considered to be stored mainly in atrial cells, its amino acid sequences are heterogeneous among species, and its proteolytic processing system is different in each species.2–4 In addition, the physiological plasma concentration of BNP is much lower than that of ANP.5 However, once the secretions of ANP and BNP are stimulated, the plasma concentration of BNP is markedly elevated and sometimes exceeds that of ANP.5 Furthermore, the increase in plasma BNP seems to be derived mainly from the ventricle.5 Similar increases in plasma and ventricular BNP were reported in hypertensive patients6 and rats with experimental hypertension.7,8 Although it is unclear which stimuli cause the ventricular expression of BNP, this phenomenon seems to be associated with cardiac hypertrophy.5–8 These findings suggest that BNP acts as an emergency hormone that assists ANP to regulate blood pressure and body fluid volume and that the synthesis and secretion of ANP and BNP may be regulated in different manners.

Accumulated evidence has suggested that both elevation of [Ca²⁺], and the activation of protein kinase C (PKC) are important for the secretion of ANP.9–15 However, it is uncertain whether these second messengers are also regulators of the synthesis of ANP. Furthermore, little is known about the intracellular mechanism involved in the synthesis and secretion of BNP. Therefore, to compare the regulation of ANP secretion and synthesis with that of BNP, we studied, using primary cultures of neonatal rat atrial cells, the follow-
Materials and Methods

Cell Culture

Atrial cells were obtained from 4-day-old Wistar rats according to the methods described previously. In brief, the atria were isolated, minced, and shaken in Ca\(^{++}\)-Mg\(^{++}\)-free Hanks' balanced salt solution with 0.8% collagenase (Wako Pure Chemical Corp., Osaka, Japan) at 37°C for four cycles of 10 minutes each. The supernatant was discarded, and cells were resuspended in culture medium consisting of 6% heat-inactivated fetal calf serum, 40% medium 199 (GIBCO Laboratories, Grand Island, N.Y.), 0.1% penicillin-streptomycin antibiotic solution, and 54% balanced salt solution containing (mM) NaCl 116, NaH\(_2\)PO\(_4\) 1.0, MgSO\(_4\) 0.8, NaHCO\(_3\) 26.2, and glucose 5. The cell suspension was diluted to 4 x 10\(^6\) cells/ml and plated on 100-mm or 60-mm culture dishes. The cells were cultured in a humidified incubator with 5% CO\(_2\)-95% air for 3-5 days at 37°C. After reaching confluence, the cultured cells were incubated for another 24 hours in serum-free medium that had the same components as the culture media described above, except that the fetal calf serum content was 0.5%. After washing the cells twice with Dulbecco's phosphate-buffered saline (GIBCO), the medium was replaced with secretion medium (complete serum-free medium supplemented with 50 µg/ml bacitracin), and incubation was performed for 1 hour with various agents, including endothelin-1 (ET), 12-O-tetradecanoylphorbol 13-acetate (TPA) with or without a PKC inhibitor, staurosporine, 1-oleoyl-2-acetylglycerol (OAG), an analogue of diacylglycerol), the calcium ionophore A23187, and Bay K 8644. After the incubation, supernatant culture media were transferred to a polypropylene tube, and the cells were washed with the secretion medium, which was then mixed with the supernatant culture media and loaded onto Sep-Pak C\(_{18}\) cartridges (Millipore Corp., Bedford, Mass.) as described below.

TFA and OAG were dissolved in dimethyl sulfoxide, Bay K 8644, and A23187 in ethanol and in ET in the culture medium. When solvents were used, they were also added to the vehicle control dishes. ET was purchased from the Peptide Institute, Osaka, Japan; TPA was from Sigma Chemical Co., St. Louis, Mo.; A23187, Bay K 8644, and bacitracin were from Wako; and OAG was from Molecular Probes, Inc., Eugene, Ore.

Extraction and Measurement of ANP and BNP

ANP and BNP in the supernatant culture media were extracted using Sep-Pak C\(_{18}\) cartridges. The media were loaded onto the cartridges equilibrated with saline, and the cartridges were washed with 0.1% trifluoroacetic acid (TFA). ANP and BNP were eluted with 60% CH\(_3\)CN containing 0.1% TFA. After evaporation, the extracts were reconstituted with the buffer for radioimmunoassay (RIA), and ANP and BNP that originated from the same cultured cells were measured at the same time. When measuring immunoreactive (ir-) ANP and ir-BNP in cultured cells, the cells were scraped off from culture dishes with a rubber policeman and boiled for 10 minutes in 5 ml distilled water to inactivate intrinsic proteases and to avoid cleavage at acid-labile peptide bonds such as Asp-Pro. After cooling, glacial acetic acid was added (final concentration, 1 M), and the cells were homogenized with a Polytron mixer for 4 minutes. The homogenates were centrifuged at 16,000g for 20 minutes, and the supernatants, which were diluted twofold with distilled water, were loaded onto Sep-Pak C\(_{18}\) cartridges equilibrated with 0.1% TFA. After washing the column with 0.1% TFA, ANP and BNP were eluted with 60% CH\(_3\)CN containing 0.1% TFA.

Under these conditions, the effects of incubation for 1 hour in the culture dish on the recovery of peptides

Figure 1. Bar graph showing secretion rates into the culture media of immunoreactive atrial natriuretic peptide (ir-ANP) stimulated with endothelin-1 (ET), 12-O-tetradecanoylphorbol 13-acetate (TPA), A23187, and Bay K 8644.
Figure 2. Bar graph showing secretion rates into the culture media of immunoreactive brain natriuretic peptide (ir-BNP) stimulated with endothelin-1 (ET), 12-O-tetradecanoylphorbol 13-acetate (TPA), A23187, and Bay K 8644.

were also analyzed. Preliminarily, γ-ANP, γ-BNP, and BNP-45 prepared from rat auricles were separated by use of a Sephadex G-50 gel filtration column (1.8x135 cm, Pharmacia LKB Biotechnology Inc., Piscataway, N.J.), and the fractions corresponding to each peptide were evaporated. These peptide fractions and synthetic α-ANP (500 fmol ANP and 150 fmol BNP) dissolved in the secretion medium were incubated in culture dishes for 1 hour, and then each peptide was subjected to RIA after extraction with a Sep-Pak C18 cartridge. The same amount of each peptide dissolved in the secretion medium was also extracted with the cartridge without a 1-hour incubation in the culture dishes and subjected to RIA as a control. Then the recovery of each peptide was calculated by comparing the amount obtained with or without incubation for 1 hour in the culture dish.

ANP and BNP in supernatant culture media and in cells were measured by RIA as reported previously.2,17

Figure 3. Bar graphs showing effects of staurosporine (ST) on 12-O-tetradecanoylphorbol 13-acetate (TPA)–stimulated secretion of immunoreactive atrial natriuretic peptide (ir-ANP, panel a) and immunoreactive brain natriuretic peptide (ir-BNP, panel b).
RIA buffer consisted of 50 mM sodium phosphate (pH 7.4), 0.25% bovine serum albumin, 0.1% Triton X-100, 80 mM NaCl, 25 mM EDTA, and 0.05% NaN₃. Peptide standards or samples were preincubated with antisera against each peptide for 24 hours; after which, ¹²⁵I-ANP or ¹²⁵I-BNP was added and incubated for another 36 hours. Free and bound tracers were separated by the double-antibody method using goat anti-rabbit y-globulin. This RIA system for ANP and BNP showed less than 0.01% cross-reactivity with each other.

Characterization of ir-ANP and ir-BNP

Both supernatant culture media and cell extracts in 0.5 M acetic acid were loaded onto a Sep-Pak C₁₈ cartridge and eluted with 60% CH₃CN containing 0.1% TFA. After evaporation, the extracts were dissolved in 1 M acetic acid and subjected to Sephadex G-50 gel filtration. As described above, y-ANP, y-BNP, and BNP-45 prepared from rat auricles and synthetic α-ANP were dissolved in 1 M acetic acid and loaded onto the same column, and elution positions of each peptide were determined. The recovery of all the peptides through this column was more than 90%. An aliquot of each fraction was subjected to RIA for ANP and BNP. Fractions exhibiting ANP and BNP immuno-reactivity were further analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC was carried out on a Hi-pore RP-318 column (4.6×250 mm, Bio-Rad Laboratories, Richmond, Calif.) with a linear gradient elution of CH₃CN from 0% to 60% in 0.1% TFA. Aliquots of all fractions were subjected to RIA for ANP and BNP.

**RNA Extraction and Northern Blot Analysis**

After incubation in serum-free media for 24 hours, the cultured atrial cells were incubated with or without 10⁻⁷ M ET, 5×10⁻⁵ M OAG, and 10⁻⁶ M A23187 for 1, 4, 8, and 16 hours. At least three experiments were performed for each stimulation. RNA was extracted from cultured cells by the acid guanidinium thiocyanate–phenol–chloroform extraction method. The extracted RNA was quantified spectrophotometrically by absorption at 260 nm. Total RNA (20 μg) denatured by glyoxal was fractionated on 1.5% agarose gels and transferred onto a nylon membrane (Zeta-probe blotting membrane, Bio-Rad). Ethidium bromide staining revealed that an equal amount of RNA was loaded on each lane. After fixation with an ultraviolet cross-linker (Stratalinker 1800, Stratagene Inc., La Jolla, Calif.), the membrane was prehybridized for 3 hours at 37°C with 6× SSPE (20× SSPE contains 3 M NaCl, 0.2 M NaH₂PO₄, and 0.02 M EDTA) containing 50% formamide, 5× Denhardt’s solution,
0.5% sodium dodecyl sulfate, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out at 37°C for 24 hours using full-length complementary DNA of rat ANP and BNP labeled with 32P as probes. After washing, the membrane was exposed to x-ray film with intensifying screens at −80°C for several days. The amounts of ANP and BNP mRNA were quantified using an LKB 2202 Ultrascan Laser Densitometer (Pharmacia LKB Biotechnology).

Statistical Analysis

Values are expressed as mean ± SEM. Effects of agents tested on ANP or BNP secretion were assessed by one-way analysis of variance. Results were considered statistically significant at p < 0.05.

Results

The spontaneous secretion of ANP and BNP into the culture supernatant increased linearly for 4 hours (ANP: 786 ± 112 [1 hour], 1,198 ± 152 [2 hours], and 1,868 ± 271 [4 hours] fmol/mg protein; BNP: 145 ± 7 [1 hour], 312 ± 34 [2 hours], and 584 ± 13 [4 hours] fmol/mg protein). In addition to choosing a time that minimized the degradation of peptides, we selected the incubation time when the agents tested were the most effective. It has been reported that the peak of phenylephrine-stimulated ANP secretion is observed between 15 and 60 minutes, and that of ET is observed at approximately 30 minutes. Furthermore, the effect of TPA was decreased at 2 hours after stimulation.13 Thus, we stimulated the cultured atrial cells for 1 hour. The recovery rate after incubation for 1 hour was 84% for α-ANP, 81% for γ-ANP, 73% for BNP-45, and 75% for γ-BNP. The recovery rate of γ-ANP and γ-BNP through Sep-Pak C18 cartridges was approximately 65%, whereas the rates for α-ANP and BNP-45 were 90% and 80%, respectively.

ET, TPA, A23187, and Bay K 8644 stimulated the secretion of ir-ANP into supernatant culture media in a dose-dependent manner. As shown in Figure 1, 10−7 M ET (540 ± 43 versus 868 ± 50 fmol/hr per milligram protein, p < 0.01), 10−5 M TPA (685 ± 37 versus 1,111 ± 73 fmol/hr per milligram protein, p < 0.01), 10−6 M A23187 (574 ± 35 versus 1,117 ± 80 fmol/hr per milligram protein, p < 0.001), and 10−6 M Bay K 8644 (599 ± 41 versus 802 ± 27 fmol/hr per milligram protein, p < 0.01) all stimulated the secretion rate of ir-ANP into the culture media when compared with the secretion rate of vehicle controls. On the other hand, ET (94 ± 3 [at 0 M] versus 148 ± 8 [at 10−7 M] fmol/hr per milligram protein, p < 0.01) and TPA (102 ± 2 [at 0 M] versus 157 ± 5 [at 10−6 M] fmol/hr per milligram protein, p < 0.001) also increased the secretion rate of ir-BNP into the culture media in a dose-dependent manner. However, A23187 (130 ± 4 [at 0 M] versus 71 ± 3 [at 10−6 M] fmol/hr per milligram protein, p < 0.001) significantly inhibited the secretion of ir-BNP into the culture media at the higher concentrations, and Bay K 8644 (141 ± 6 [at 0 M] versus 152 ± 5 [at 10−6 M] fmol/hr per

Figure 5. Panel a: Analysis by reverse-phase high-performance liquid chromatography (RP-HPLC) of high molecular weight immunoreactive (ir-) atrial natriuretic peptide (ANP) in culture media (upper row) and in cells (lower row). Panel b: Analysis by RP-HPLC of low molecular weight ir-ANP in culture media. Panel a represents fractions 25–31 (131 μl per tube) in Figure 2a and fractions 24–31 (31 μl per tube) in Figure 2b. Panel b represents fractions 46–56 (105 μl per tube) in Figure 2a. Conditions were as follows: column, Hi-pore RP-318 (4.6x250 mm, Bio-Rad Laboratories, Richmond, Calif.); solvent A, H2O:CH3CN:10% TFA at 100:0:1; solvent B, H2O:CH3CN:10% TFA at 40:60:1; linear gradient elutions from A to B, 80 minutes. Arrows indicate the elution positions of rat α-ANP (1) and rat γ-ANP (2).
milligram protein, \( p=NS \) did not exert significant effects on BNP secretion (Figure 2).

To determine whether or not the TPA-induced increases in ANP and BNP secretion were mediated by PKC activation, the effects of PKC inhibitor staurosporine were examined. As shown in Figure 3, staurosporine suppressed the TPA-induced secretion of ANP (955 ± 164 [10^{-6} M TPA] versus 407 ± 6 [10^{-6} M TPA + 10^{-8} M staurosporine] fmol/hr per milligram protein, \( p<0.05 \)) and BNP (148 ± 7 [10^{-6} M TPA] versus 76 ± 1 [10^{-6} M TPA + 10^{-8} M staurosporine] fmol/hr per milligram protein, \( p<0.001 \)) in a dose-dependent fashion.

Figure 4 shows the gel filtration profile in the non-stimulated condition. In supernatant culture media, two peaks of ir-ANP were eluted in fractions of molecular weight (MW) 11,000–13,000 and 3,000 (Figure 4a), but in cultured cell extracts, ir-ANP was eluted only in fractions of 11,000–13,000 (Figure 4b). The ratio of the high and low MW ir-ANP in the supernatant culture media was approximately 1:1. As shown in Figure 5, further analysis by RP-HPLC confirmed that the high and low MW of ir-ANP corresponded to \( \gamma \)-ANP and \( \alpha \)-ANP, respectively. Although small amounts of low MW ir-ANP in the culture media were eluted at earlier times than for \( \alpha \)-ANP, they were regarded as degraded compounds of \( \alpha \)-ANP. On the other hand, ir-BNP was detected as two peaks, MW 11,000–13,000 and 5,000, both in culture media and cell extracts (Figure 4). The retention times of these two peaks in RP-HPLC were identical to those of \( \gamma \)-BNP and BNP-45 (Figure 6).

Small portions of high MW ir-BNP in cultured cells emerged at earlier times than did \( \gamma \)-BNP. They were also considered to be \( \gamma \)-BNP with different intramolecular conformations, because, when boiled, large peptides are denatured, resulting in productions of peptides with the identical primary structure but with different conformations. The similar phenomenon was observed when human \( \gamma \)-BNP was isolated.

Both the secretion rate into supernatant culture media and cell contents of ir-ANP and ir-BNP in basal states were calculated after gel filtration. The ratio of the secretion rate (per hour) to cell contents was approximately 1:9 for ir-ANP (1.0 pmol/hr per milligram protein versus 9.1 pmol/mg protein) and approximately 1:1 for ir-BNP (96.9 fmol/hr per milligram protein versus 120.1 fmol/mg protein).

Figures 7 and 8 present autoradiographs of ANP mRNA and BNP mRNA, respectively, which were obtained after stimulation with the agents. The amount of BNP mRNA was small, probably because of its instability due to its AU-rich regions at the 3' untranslated portion. As shown in Figure 9, both ET and OAG significantly increased the accumulation of ANP mRNA at 8 hours after stimulation (\( 10^{-7} \) M ET, \( +52 \pm 13 \)%, \( p<0.05 \); \( 5 \times 10^{-7} \) M OAG, \( +296 \pm 43 \)%, \( p<0.01 \)) whereas A23187 did not produce a significant increase in ANP mRNA at any period. ET and OAG significantly enhanced the accumulation of BNP mRNA at 16 and 8 hours after stimulation, respectively (\( 10^{-7} \) M ET, \( +36 \pm 9 \)%, \( p<0.02 \); \( 5 \times 10^{-7} \) M OAG, \( +133 \pm 8 \)%, \( p<0.001 \)). In contrast, A23187 had no significant effect on BNP mRNA at any time.

Discussion

It has been indicated that ANP is secreted from the cardiac atria through a regulated pathway, whereas ANP from the cardiac ventricles is secreted through a constitutive pathway. Bloch et al. found a higher ratio of secreted ANP to cellular ANP in cultured ventricular cells than in atrial cells. They also found much more ANP-containing granules in the atria than in the ventricles. Furthermore, they showed by pulse-chase analysis that a larger amount of ANP was secreted rapidly after synthesis in the ventricles than in the atria. However, it remains to be elucidated whether or not BNP in the cardiac atria is secreted via a regulated pathway. Using an immunocytochemical method, Hase-
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Figure 7. Northern blot analysis of atrial natriuretic peptide mRNA extracted from cultured atrial cells that were stimulated by \(10^{-7}\) M endothelin-1 (panel a), \(5 \times 10^{-5}\) M 1-oleoyl-2-acetyl-glycerol (panel b), and \(10^{-6}\) M A23187 (panel c). C, control; S, stimulation. Ten micrograms total RNA (adult rat atria) or 20 \(\mu\)g total RNA (cultured atrial cells) was loaded onto each lane.

gawa et al23 examined the localization of ANP and BNP in porcine atria and found that there were two types of granules. Type 1 was a monohormonal granule containing ANP alone, and type 2 was a multihormonal granule containing both ANP and BNP. They also found differences in the localization and distribution patterns between type 1 and 2 granules: type 1 granules were mainly distributed on the epicardial side, whereas a major portion of type 2 granules was located on the subendocardial side. Furthermore, ANP was distributed diffusely in almost all atrial cells, whereas BNP formed foci of BNP-containing cells in the subendocardium. These results indicate that some atrial myocytes do not synthesize BNP or do not store BNP in the granules. In the present study, the ratio of the secretion rate to cell contents in cultured atrial cells was much higher in BNP than in ANP. Taking these data into account, our results indicate that the regulatory mechanism for the storage of these peptides is different and that BNP has a tendency to be secreted soon after its synthesis, although some synthesized BNP may be stored in a small subset of atrial granules.

ANP and BNP showed the contrasting response to [Ca\(^{2+}\)]-increasing agents such as A23187 and Bay K 8644. A23187 suppressed BNP secretion at higher concentrations. However, it seems unlikely that the marked elevation in [Ca\(^{2+}\)] by A23187 may cause some toxic effects, because it increased ANP secretion from the same cells. Moreover, LaPointe et al15 showed that cell viability was not influenced by this agent at concentrations used in our study. Therefore, it is strongly suggested that the secretion of both natriuretic peptides may be regulated by different mechanisms.

The molecular forms of ir-ANP secreted into culture media were \(\gamma\)-ANP and \(\alpha\)-ANP, and their relative contents were approximately 1:1, whereas the cultured cells contained exclusively \(\gamma\)-ANP. Early reports suggested that cultured atrial cells secrete mostly pro-ANP.23 However, other reports have since shown that approximately 60–70% of the ANP secreted into the culture medium was \(\gamma\)-ANP.24–25 Although the mechanisms for the processing of ANP in the culture medium are unclear, Shields and Glembocki26 clearly showed that processing decreased as the duration of cell culture was prolonged and that glucocorticoids enhanced this processing. In the present study, the molecular forms of the natriuretic peptides were determined in the early phase (on about day 5) of culture. Our findings are compatible with those reported previously when taking into consideration that the recovery of \(\gamma\)-ANP by SepPak C\(_{18}\) extraction was lower than that of \(\alpha\)-ANP. On the other hand, the molecular forms of ir-BNP, both secreted into culture media and stored in cells, were \(\gamma\)-BNP and its C-terminal 45-amino-acid peptide, BNP-45. Their relative contents were approximately 2:5 in culture media and approximately 1:2 in cells. It has been reported that the ratio of \(\gamma\)-BNP to BNP-45 was also approximately 1:3 in the atria of adult rats2 and that BNP-45 is a major storage form in atrial tissue.2,27 These findings are compatible with our results in neonatal rat cultured atrial cells. The difference in the major molecular form between ANP and BNP suggests that these peptides are processed in different manners in cultured atrial cells.

We examined the recovery rates of high and low molecular weight forms of the peptides from culture dishes after incubation. The recovery of all the peptides was more than 70%, and differences between the high and low molecular weight forms were relatively small. Furthermore, we estimated that the ratio of the ANP content in the medium to that in the cells was approximately 10%, which was a ratio similar to that described by Shields and Glembocki26 (7–15%) and by Bloch et al20 (14%). Thus, nonspecific adsorption of each peptide to the culture dish and cells does not seem to affect our interpretation of the results. We cannot exclude the possibility that \(\gamma\)-ANP and \(\gamma\)-BNP may be specifically converted to \(\alpha\)-ANP and BNP-45, respectively, after being secreted into the medium. Such conversion could influence our interpretation of the results. However, the intracellular molecular forms of ANP and BNP were different, with ANP being exclusively the prohormone, whereas both the prohormone and mature forms of BNP were detected. It seems unlikely that only BNP would be processed during our extraction procedure. In addition, our data were consistent with the composition of molecular forms of ANP and BNP in atrial tissue in previous reports,2,17 suggesting that the methods of cell
incubation and peptide extraction used in the present study did not alter the results. The intracellular presence of the mature molecular form of BNP also suggests a tendency of BNP to be secreted soon after synthesis.

ET is known to increase the synthesis and secretion of ANP in cultured atrial cells. ET stimulates the hydrolysis of inositol phospholipids and elevates [Ca\(^{2+}\)]. The accumulation of these second messengers on the secretion and synthesis of natriuretic peptides have been scarcely examined. The accumulation of both ANP and BNP mRNA was stimulated by ET and OAG but not by A23187. This indicates that synthesis of ANP and BNP is stimulated by PKC activation rather than by the elevation of [Ca\(^{2+}\)], although it also remains possible that PKC activation inhibits the degradation of ANP and BNP mRNA. LaPointe et al. showed that both A23187 and TPA increased the secretion of ANP, whereas both in combination significantly increased it. This difference with respect to the effect of PKC activation from ours may be related to the action of TPA, which is known to downregulate PKC within 1 hour. In fact, OAG, which does not desensitize PKC, stimulated the synthesis of ANP in the present study.

It is frequently documented that cardiac hypertrophy is associated with the increased synthesis of ventricular ANP and BNP. The present data indicating that OAG stimulates the synthesis of ANP and BNP may help to explain this phenomenon. When cardiac hypertrophy is in process, the stimulation of phospholipase C-coupled receptors such as \(\alpha_1\)-adrenoceptors and angiotensin II receptors results in an increase in the production of diacylglycerol, which in turn activates PKC. It has recently been reported that ET also acts as a local promoter of cardiac hypertrophy. It is well established that atrial stretch is the most important physiological stimulus for ANP secretion. However, there is still controversy with respect to the intracellular mechanisms responsible for the stretch-induced secretion of ANP. Ruskoaho et al. showed that stretch-induced ANP secretion from the isolated heart was augmented by a PKC activator but not by a

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**Figure 8.** Northern blot analysis of brain natriuretic peptide mRNA extracted from cultured atrial cells that were stimulated by \(10^{-7}\) M endothelin-1 (panel a), \(5 \times 10^{-5}\) M 1-oleoyl-2-acetyl-glycerol (panel b), \(10^{-6}\) M A23187 (panel c). C, control; S, stimulation. Ten micrograms total RNA (adult rat atria) or 20 \(\mu\)g total RNA (cultured atrial cells) was loaded onto each lane.

**Figure 9.** Bar graphs showing changes in the amount of atrial natriuretic peptide (ANP) mRNA (panel a) and brain natriuretic peptide (BNP) mRNA (panel b) stimulated with \(10^{-7}\) M endothelin-1 (ET), \(5 \times 10^{-5}\) M 1-oleoyl-2-acetyl-glycerol (OAG), and \(10^{-6}\) M A23187. Values are expressed as percent increases at 8 hours after stimulation, except that the increase in BNP mRNA when stimulated with ET is estimated at 16 hours after stimulation. At least three experiments were performed for each stimulation.
Ca$^{2+}$ ionophore. Greenwald et al also demonstrated the negative effect of [Ca$^{2+}$] elevation on the increase in ANP release by stretch from cultured atrial cells. Furthermore, there is some discrepancy regarding the reports on the relation between [Ca$^{2+}$], and stretch-induced ANP secretion in isolated atrial tissues; i.e., ANP secretion is reported to be modulated by extracellular Ca$^{2+}$ in a positive$^{29,40}$ or a negative$^{41}$ manner. However, Ishida et al$^{42}$ have reported that inhibitors for PKC or calmodulin do not influence the stretch-stimulated ANP secretion from isolated heart preparations.

Further studies are required to elucidate the intracellular mechanisms for stretch-induced ANP secretion.

In conclusion, despite the similar natriuretic and vasodilating effects, the regulatory mechanisms for the secretion and processing of ANP and BNP are not identical. This difference seems to be related to their different secretory pathways and, in turn, the different pathophysiological roles of these natriuretic peptides.

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