Brefeldin A Defines Distinct Pathways for Atrial Natriuretic Factor Secretion in Neonatal Rat Atrial and Ventricular Myocytes

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The intracellular pathways for basal atrial natriuretic factor (ANF) secretion from the heart and their correlation with ANF processing to the active form were characterized in cultured neonatal rat atrial and ventricular myocytes. Brefeldin A, a fungal antimetabolite that blocks transport of newly synthesized proteins from the endoplasmic reticulum, was used to inhibit nascent protein trafficking. Thus, release of newly synthesized hormone was blocked, but release of stored hormone was unaffected. Whereas brefeldin A inhibited basal ventricular ANF release to 10% of the control value, basal ANF release from atrial cells was enhanced. Furthermore, basal atrial ANF secretion was inhibited by agents preventing myocyte depolarization, Ca\(^{2+}\) influx, release of Ca\(^{2+}\) from intracellular stores, or activation of protein kinase C, whereas ventricular ANF secretion was unaffected by these agents.

Atrial natriuretic factor (ANF) is a hormone secreted by the atrium, which regulates blood volume and blood pressure by stimulating receptors located in the kidney, adrenal gland, vascular smooth muscle, and discrete regions of the brain to cause diuresis, natriuresis, and vascular relaxation.\(^1\) ANF biosynthesis is similar to that of other peptide hormones, since ANF is stored in secretory granules and its release can be stimulated by appropriate secretagogues.\(^3\) However, whereas the primary physiological stimulus for ANF secretion is atrial stretch caused by volume expansion, ANF is also secreted continually, even under basal conditions. This constant basal secretion of ANF has been demonstrated both in vivo and in vitro. In primary cultures of rat atrial myocytes, the basal secretion rate of ANF per hour represents \(\approx\) 10% of the total cell ANF content.

In the present study, we investigated the cellular mechanisms underlying basal ANF secretion in cultured neonatal atrial myocytes. It is possible that this basal secretion is derived from the constitutive pathway of protein secretion, whereas stimulated ANF secretion originates from the triggered release of secretory granules. Dual mechanisms of secretion have been observed in endocrine cells such as the AtT20 pituitary cell line.\(^4\) Alternatively, basal ANF secretion may represent ANF secretory granule exocytosis stimulated by spontaneous spikes in intracellular Ca\(^{2+}\) levels associated with myocyte contraction.

Resolution of the ANF secretory pathways used by atrial cells is also necessary to clarify the relative location of pro-ANF [ANF-(1–126)] processing to the active form [ANF-(99–126)]. Although atrial myocytes cosecretionally cleave ANF to the correct circulating forms, processing is only 70% to 80% complete.\(^5\) Since maturational processing generally correlates with secretion via the regulated pathway, incomplete ANF processing by atrial myocytes could indicate that a portion of ANF is being secreted via a constitutive pathway that lacks a processing enzyme. Because an ANF processing enzyme has been isolated from bovine atrial granules,\(^6\) it is feasible that ANF processing is pathway specific. Lack of correlation of ANF processing with the regulated secretory pathway may reflect the existence of a novel cardiac-specific processing mechanism.

A parallel study of ANF secretion from neonatal ventricular myocytes was also undertaken because of recent questions raised regarding the secretory mechanisms in this cell type. That is, ventricular ANF secretion was originally believed to be primarily constitutive; this belief was based on the lack of secretory granules in this tissue\(^7\) and the inability to stimulate ANF secretion to levels above those observed basally.\(^8\) However, recent studies have demonstrated that ANF secretion from primary cultures of ventricular myocytes can be stimulated by various secretagogues,\(^9\)\(^-\)\(^11\) disputing the notion that ventricular ANF secretion is entirely constitutive.
The ability of ventricular myocytes to secrete ANF in a regulated manner may be related to the previous curious observation that these cells are able to cosecretionally cleave pro-ANF to ANF-(99–126), similar to observations in atrial myocytes. That is, neonatal ventricular myocytes may share several characteristics of the atrium as an endocrine organ beyond mere hormone expression. Thus, a further characterization of ventricular ANF biosynthesis and secretion was necessary.

To distinguish between constitutive ANF secretion and constant release of ANF from secretory granules, which is possibly due to second-messenger signals associated with cell beating, we used the fungal antimitabolite brefeldin A. This compound inhibits secretion in many cell types in different species by blocking transport of newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus. This effect is reported to be rapidly reversed on removal of the drug, resulting in less cellular toxicity than protein synthesis inhibitors. Because transport of proteins to the Golgi apparatus is necessary for packaging of peptide hormones into either secretory granules or constitutive transport vesicles, brefeldin A should prevent both the regulated and constitutive secretion of newly synthesized ANF. However, secretion via the regulated pathway of ANF stored in secretory granules should be unaffected by brefeldin A. The regulation, or lack thereof, of basal ANF secretion by second-messenger signals was also used to distinguish between constitutive versus regulated ANF secretion because the rate of constitutive secretion, by definition, is constant and unresponsive to any signaling pathway.

Additionally, the effect of brefeldin A on maturational processing was examined. If ANF processing is correlated with either secretory pathway, the ratio of ANF-(1–126) to ANF-(99–126) secreted into the culture medium should change with inhibition of secretion by brefeldin A.

The results of the present study extend previous observations revealing marked differences in ANF secretory pathways and mechanisms between atrial and ventricular cells and demonstrating that the ANF processing enzyme is active in either secretory pathway.

Materials and Methods

Preparation of Neonatal Rat Myocytes in Culture

Cardiac cells were prepared according to the procedure of Fischman et al. In brief, 1- to 3-day-old Sprague-Dawley rats were decapitated after chloroform vapor anesthesia, and their hearts were removed. The atria and ventricles were separated by dissection. The heart tissues were then digested in a solution of 0.25% porcine trypsin in Ca2+- and Mg2+-free buffered saline with 0.2% EDTA and supplemented with 58 U/mL trypsin. When microscopic examination revealed isolated cells in the medium, the medium was collected, and the tissue was placed in fresh trypsin solution. This process was repeated three to four times until viable (rod-shaped) cells were no longer released from the tissue. The cell-containing trypsin solutions were mixed with a sterile solution of 1:1 DMEM/F-12 containing 10% fetal bovine serum (FBS) and antibiotics. After sedimenting the cells (50g for 5 minutes) and resuspending them in fresh DMEM/F-12 plus FBS medium, they were preplated twice (2 hours each) to remove fibroblasts from the cell suspension on the basis of the preferential attachment of fibroblasts. Final plating density was 2 to 5x10^5 myocytes per uncoated 16-mm well.

Measurement of Cell Secretion of ANF

Both the amount of ANF secreted from the cells and cellular ANF content were determined for each culture well. At the time of the experiment, CSFM was removed from the cells and replaced with "secretion medium" consisting of DMEM/F-12 supplemented only with 0.25% BSA, 0.7% fructose, insulin, and antibiotics. The cells were incubated in 0.5 mL of this solution with or without added agents at 37°C for 1 hour, and then the medium was collected. In experiments including brefeldin A, the cells were preincubated in secretion medium containing 5 µg/mL brefeldin A for 30 minutes before changing to fresh secretion medium also containing brefeldin A for collection. After removal of the medium, the cells were harvested in 0.5 mL of a "lysis buffer" consisting of 0.5 mol/L NaCl, 1% Nonidet P-40, and 10 mmol/L HEPES, pH 7.4. Phenylmethylsulfonyl fluoride (PMSF, 17 µg/mL) was added to prevent proteolysis during storage.

Medium and cellular ANF levels were measured by radioimmunoassay according to standard procedures using either anti-ANF-(103–126) (commercially available anti-ANF-(99–126) antibody purchased from Amersham, Arlington Heights, Ill.) or anti-ANF-(99–126) purchased from Amersham, Arlington Heights, Ill. Both antibodies recognize the C-terminus, detecting pro-ANF and ANF-(99–126). Peninsa antisemurs detects as little as 1 pg ANF-(99–126), whereas the other antisemurs detects 50 pg ANF-(99–126). The data are expressed as the ratio of ANF in the medium (in picograms) to the total amount of ANF in the well (picograms in the medium plus picograms in the cells) to normalize for differences between culture wells. Atrial walls contained an average of 41±33 ng ANF per well (mean±SD, 14 experiments), and ventricular walls averaged 12±8 ng ANF per well (8 experiments).

Statistically significant changes in the amounts of secretion were calculated using paired Student's t test as described by Zar. Data are expressed as mean±SEM.

Metabolic Labeling of Newly Synthesized Cell Proteins with [35S]Cysteine

For identification of newly synthesized proteins, the myocytes were metabolically labeled with [35S]cysteine. This procedure allowed brefeldin A activity to be detected as a reduction in release of [35S]-labeled proteins into the cell medium, which results from the trapping of newly synthesized proteins in the endoplasmic reticulum–Golgi composite produced by brefeldin A.
Before addition of the [³⁵S]cysteine, the cells were preincubated for 30 minutes at 37°C in RPMI medium containing a low (40 μmol/L) concentration of cysteine and supplemented with 0.25% BSA, 0.70 μmol/L dexamethasone, and antibiotics. Subsequently, the preincubation medium was replaced with 0.5 mL of low-cysteine RPMI containing 100 to 250 μCi/mL [³⁵S]cysteine (specific activity, 1000 Ci/mmol). The cells were metabolically labeled for 1 hour after removal of the radioactive medium, secretion medium was added to the cells and collected after 1 hour. In experiments involving brefeldin A, 5 μg/mL brefeldin A was added to the low-cysteine medium and included in the preincubation, labeling, and secretion steps.

Metabolically, [³⁵S]labeled proteins were analyzed in several ways. For specific examination of [³⁵S]labeled ANF, anti-ANF-(103–126) antibody was used in an immunoprecipitation procedure described by Wilday et al. In this assay, an immune complex was initially formed by incubation of a 10-μL aliquot of undiluted antiserum with 40 μg protein A–sepharose in 0.5 mL of "radioimmunoassay buffer" (50 mmol/L NaCl, 0.1% Triton X-100, 0.1% BSA, and 100 mmol/L phosphate, pH 7.4) for 2 hours on ice. After sedimentation of the complex (500g, 5 minutes) and resuspension in the same volume of medium, 0.5 mL of ANF-containing secretion medium or cell lysate was combined with the preformed immune complex. This mixture was incubated overnight at 5°C, followed by sedimentation (500g, 5 minutes) and then resuspension of the pellet with lysis buffer. After centrifugation (500g), the final immunoprecipitated complexes were analyzed by electrophoresis (see below). There are only two cysteines in the entire pro-ANF molecule, which are located at C-terminal residues 105 and 121. Thus, the pro-ANF and mature ANF forms will have the same number of labeled cysteines.

In some experiments, total [³⁵S]labeled proteins released into the secretion medium and those remaining in the cells were examined. This was accomplished either by direct electrophoresis of 50 to 100 μL of cell secretion medium or cell lysate (Fig) or by a trichloroacetic acid (TCA) precipitation procedure. TCA was added to the cell secretion medium or directly to cells in a culture well to a final concentration of 10% (wt/vol). After a 10-minute incubation on ice, the TCA-cell mixtures were centrifuged at 10 500g for 5 minutes in a Sorvall Microspin centrifuge to sediment the proteins. After removal of the supernatants and two rapid washes of the pellet with ice-cold 5% TCA, the portion of the 1.5-mL microcentrifuge tube containing the pellet was cut directly into Ecoscint scintillation fluid (National Diagnostics, Manville, NJ) and counted in a Beckman LS 6000IC scintillation counter. Quantities of TCA-precipitable [³⁵S]labeled proteins in the medium with and without added brefeldin A were determined.

**Electrophoresis**

Electrophoretic separation of [³⁵S]labeled proteins was accomplished using a variation of a Laemmli sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) system recommended by the Hoefer Scientific catalogue for low-molecular-weight proteins. Briefly, a 20% polyacrylamide gel prepared with 0.5% cross-linking and 0.1% SDS and including 10% (vol/vol) glycerol with 0.75 mol/L Tris at pH 9.3 was used for the separation. This resulted in sharper resolution of the 3-kD ANF-(99–126) band. Gels were run overnight at 13 mA per gel (1.5-mm thickness in a Hoefer SE 600 vertical slab unit). After soaking for 1 hour in Autorfluor (National Diagnostics), they were dried in a 42°C oven suspended in a frame between two sheets of cellophane membrane backing (Bio-Rad Laboratories, Richmond, Calif).

Cell lysates, in which no ANF-(99–126) was observed, were subjected to electrophoresis using 15% acrylamide gels prepared analogously to the above.

**High-Performance Liquid Chromatography of Secretory Samples to Separate ANF-(1–126) From ANF-(99–126)**

High-performance liquid chromatography (HPLC) was performed to separate unprocessed from processed ANF as described previously. Secretory medium was mixed 1:3 with 0.1% trifluoroacetic acid (TFA) on ice and centrifuged at 10 500g for 5 minutes to precipitate proteins before injection into a Beckman model 110A HPLC system equipped with a Vydac C₄ column. The ANF peptides were eluted at 1 mL/min with a gradient increasing from 30% to 55% in 0.1% TFA acetonitrile over 30 minutes. One-milliliter fractions were collected. The ANF-(99–126) peak eluted at fraction 19; pro-ANF eluted at fraction 29. These peaks were identified.
both by radioimmunoassay of the HPLC fractions and by comparison of their elution with ANF standards. In studies investigating the processing of pro-ANF after addition to culture wells, the $^{35}$S-labeled pro-ANF used for the studies was obtained by HPLC purification after immunoprecipitation from ventricular cell lysates. The fractions eluted from the HPLC column that contained pro-ANF were dried in a Savant Speed Vac Concentrator and resuspended in secretion medium.

**Chemicals**

Brefeldin A was purchased from Epicentre Technologies, Madison, Wis. Veratridine, BSA, protein A–sepharose, dexamethasone, and other tissue culture reagents and protease inhibitors were obtained from Sigma Chemical Co, St Louis, Mo. FBS was purchased from Whittaker Biosciences, Walkersville, Md. TPA, endotheIin, and staurosporine were from Calbiochem Co, San Diego, Calif. The Ca$^{2+}$- and Mg$^{2+}$-free 0.25% porcine trypsin solution was purchased from JRH Biosciences, Lenexa, Kan. Supplementary trypsin solution was from Worthington Biochemical Corp, Freehold, NJ. $^{35}$S-Cysteine was from ICN Biomedicals Inc, Irvine, Calif. Cysteine-free RPMI was made from a Select-Amine kit from GIBCO BRL, Grand Island, NY.

**Results**

**Verification of Brefeldin A Activity**

Initial experiments involved establishing that brefeldin A would block the secretion of newly synthesized proteins from neonatal rat cardiac cells. Previous experiments had shown that the characteristic morphological changes in the endoplasmic reticulum associated with brefeldin activity were also observed in atria. To verify changes in protein transport, new protein synthesis was monitored by $^{35}$S-cysteine labeling followed by SDS-PAGE of the tissue culture medium and cells. These experiments demonstrated that concentrations of brefeldin A from 0.1 to 5 $\mu$g/mL decreased the amount of $^{35}$S-labeled proteins secreted into the medium. The results of incubating $^{35}$S-labeled atrial cells with 5 $\mu$g/mL brefeldin A, the maximally effective dose used in subsequent experiments, are given in the Figure. This concentration of drug markedly reduced the amount of $^{35}$S-labeled protein secreted into the culture medium (panel A) but produced little apparent change in cellular protein synthesis (panel B). Similar results were observed with ventricular cells. The results also verify cell intactness. If cell integrity were altered by brefeldin A, more $^{35}$S-labeled proteins should have been visible in the culture medium.

To quantify the observed effect of brefeldin A, $^{35}$S-labeled proteins in both the culture medium and cells were precipitated with TCA and counted for radioactivity. As shown in Table 1, this method again demonstrated that total secretion of $^{35}$S-labeled protein was significantly reduced by brefeldin A treatment. However, this was associated with a small change in apparent protein synthesis. Brefeldin A decreased the incorporation of $^{35}$S into total cellular protein by 22±3% (mean±SEM, n=4, P<.01). This observation is consistent with the results of a previous study showing that effective doses of brefeldin A produced a small decrease in $^{35}$S-labeled total protein. In the previous study, a dose of 10 $\mu$g/mL brefeldin A decreased apparent protein synthesis in cultured rat hepatocytes by 26%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Atrial</th>
<th>Ventricular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ANF secretion, pg ANF (medium)/pg total ANF (well)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (basal)</td>
<td>0.14±0.03</td>
<td>0.38±0.04t</td>
</tr>
<tr>
<td>5 $\mu$g/mL Brefeldin</td>
<td>0.19±0.05</td>
<td>0.05±0.01t</td>
</tr>
<tr>
<td>Release of TCA-precipitable $^{35}$S-labeled proteins, % control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (basal)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5 $\mu$g/mL Brefeldin</td>
<td>8±0*</td>
<td>12±3*</td>
</tr>
</tbody>
</table>

ANF indicates atrial natriuretic factor; TCA, trichloroacetic acid. Values are mean±SEM. ANF secretion is presented as picograms ANF released into the medium per picograms total ANF per well as determined by radioimmunoassay (n=4).

The effects of brefeldin A treatment specifically on ANF biosynthesis and secretion were similar to the effects on total protein synthesis and release. Brefeldin A markedly reduced the amount of immunoprecipitable $^{35}$S-labeled ANF secreted into the culture medium (panel C). This was associated with a small but significant decrease in the total ANF content of myocyte culture wells (19±3%, mean±SEM, n=6, P<.01) as measured by radioimmunoassay. It was also observed that inhibition of ANF secretion was partially reversed on removal of the drug (panel C).

One possible explanation for the incomplete reversal of brefeldin A action, as well as its effect on protein synthesis, is that the proteins trapped in the endoplasmic reticulum are partially degraded over time. This is consistent with our observation that more $^{35}$S-labeled proteins migrated with the dye front on SDS-PAGE gels of brefeldin A–treated samples compared with untreated control samples. Nevertheless, the small effect of brefeldin A on total myocyte protein content is insufficient to account for the observed changes in secretion. Therefore, our results are consistent with the proposed mechanism of action of brefeldin A, which is to block trafficking of nascent proteins.

**ANF Secretory Pathways**

The effects of brefeldin A on total ANF secretion from atrial and ventricular cells were determined by radioimmunoassay and are shown in Table 1. Under control conditions, 14% of total atrial ANF was secreted into the medium in 1 hour. Ventricular cells secreted 38% of their total ANF per hour, revealing less hormone storage. In the presence of brefeldin A, ventricular secretion was markedly reduced; however, no inhibition of atrial secretion was observed. In fact, in 10 experiments performed on atrial cells, ANF secretion was increased to 146±50% of the control (mean±SD, P<.01) by the addition of brefeldin A, although reduced release of $^{35}$S-labeled proteins in the same cell wells verified blockade of protein trafficking. However, the extent of this enhancement was variable, leading to less significance in small data pools (as seen in Table 1).
**Table 2. Effect of Inhibitors of Signaling on Basal Atrial Natriuretic Factor Secretion**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ANF Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>10 μmol/L Saxitoxin</td>
<td>0.09±0.02*</td>
</tr>
<tr>
<td>50 μmol/L Pinacidil</td>
<td>0.05±0.01†</td>
</tr>
<tr>
<td>3 mmol/L EGTA</td>
<td>0.06±0.01†</td>
</tr>
<tr>
<td>20 μmol/L Verapamil</td>
<td>0.06±0.02‡</td>
</tr>
<tr>
<td>10 μmol/L Ryanodine</td>
<td>0.07±0.02*</td>
</tr>
<tr>
<td>3 mmol/L EGTA + 1 μmol/L ryanodine</td>
<td>0.03±0.01*</td>
</tr>
<tr>
<td>100 mmol/L Staurosporine</td>
<td>0.05±0.01†</td>
</tr>
<tr>
<td>50 μmol/L Pinacidil + 100 nmol/L staurosporine</td>
<td>0.04±0.01*</td>
</tr>
<tr>
<td>3 mmol/L EGTA + 100 nmol/L staurosporine</td>
<td>0.04±0.02*</td>
</tr>
<tr>
<td>3 mmol/L EGTA + 50 μmol/L pinacidil + 100 nmol/L staurosporine</td>
<td>0.04±0.01*</td>
</tr>
</tbody>
</table>

ANF indicates atrial natriuretic factor. ANF secretion is presented as picograms ANF released into medium per picograms total ANF per well. Values are mean±SEM (n=2 to 4 independent studies for each inhibitor).

*P<.025 vs control.
†P<.01 vs control.
‡P<.05 vs control.

**Table 3. Effect of Brefeldin A on Atrial and Ventricular Processing of Pro–Atrial Natriuretic Factor (Atrial Natriuretic Factor-[1–126]) to Atrial Natriuretic Factor-(99–126)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ANF Processing*, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial</td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>80±4</td>
</tr>
<tr>
<td>5 μg/mL Brefeldin</td>
<td>74±2</td>
</tr>
<tr>
<td>100 nmol/L Endothelint</td>
<td>85±2</td>
</tr>
<tr>
<td>5 μg/mL Brefeldin + 100 nmol/L endothelint</td>
<td>84±3</td>
</tr>
<tr>
<td>Ventricular</td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>70±7</td>
</tr>
<tr>
<td>5 μg/mL Brefeldin</td>
<td>76±4</td>
</tr>
</tbody>
</table>

ANF indicates atrial natriuretic factor. Values are mean±SEM (n=3 independent studies per treatment).

*Processing of pro-ANF to ANF-(99–126) was determined using high-performance liquid chromatography as detailed in “Materials and Methods.”
†Endothelin increased ANF secretion by 188% over control.

**Regulation of Atrial ANF Secretion**

The level of regulation of the “spontaneous” atrial ANF secretion process was investigated next. Specifically, we investigated whether or not basal ANF release is an unregulated phenomenon due to ongoing granule fusion and hormone release or a regulated event correlated with cell signals associated with myocyte beating. To examine this issue, a variety of signaling inhibitors was added to the cells to block ANF release. An absence of response to the inhibitors would suggest unregulated granule release; inhibition would suggest a role for these messengers in basal ANF secretion.

Many agonists that stimulate ANF secretion either activate protein kinase C or increase cytosolic [Ca²⁺]. As shown in Table 2, the protein kinase C inhibitor staurosporine, as well as a variety of compounds that prevent increased intracellular [Ca²⁺], such as saxitoxin (which blocks voltage-dependent sodium channels), pinacidil (which opens ATP-dependent potassium channels), EGTA (which chelates extracellular Ca²⁺), verapamil (which blocks Ca²⁺ and Na⁺ channels), and ryanodine (which depletes sarcoplasmic reticulum Ca²⁺ stores), all inhibited basal ANF secretion. The concentrations of the compounds used in these studies were the minimal concentrations shown by us²²⁻²⁴ and others²⁵⁻²⁶ to specifically inhibit the desired signaling pathway. None of the added agents altered the total amount of ANF synthesized in a given atrial culture well.

Adding multiple agents affecting both the Ca²⁺ and protein kinase C secretory mechanisms (ie, EGTA, pinacidil, and staurosporine) did not inhibit secretion below a level of ~4% of total synthesized ANF, even in the presence of brefeldin A (0.04±0.01 pg ANF [medium] per picogram ANF [culture well] in control cells versus 0.05±0.01 pg ANF [medium] per picogram ANF [culture well] with brefeldin A, n=2). This indicates that the agents are acting incompletely or that a small fraction of ANF release via granules is unregulated but not due to constitutive ANF secretion.

In parallel experiments, the same agents were added to ventricular cells, and neither EGTA, pinacidil, staurosporine, nor these agents added in combination decreased ANF secretion significantly. Thus, the secretory pathway used for release of ventricular ANF is not regulated by the intracellular signals important to atrial ANF secretion.

**Correlation of Pro-ANF Processing and the Secretory Pathway**

As shown in Table 3, 70% to 80% of atrial and ventricular myocyte ANF is secreted as the processed mature form of the hormone, ie, ANF-(99–126). This was determined both by SDS-PAGE (Figure) and by HPLC analyses of the culture medium (see “Materials and Methods”). Atrial ANF processing was unaffected by the addition of brefeldin A or by stimulation of secretion by endothelin (Table 3). These findings suggest that both basal and “stimulated” ANF secretion from atrial cells are derived from the same secretory pathway. The ANF processing of ventricular myocytes was not significantly different from atrial processing, even when total secretion was inhibited to 10% by brefeldin A, suggesting that pro-ANF processing does not require passage through the regulated pathway. Ventricular processing of pro-ANF has also been observed by Glembotski et al.²⁵⁻²⁶

There was no evidence of pro-ANF processing by the culture medium itself or by any other cell types in the culture. When trace amounts of exogenously added [³⁵S]-labeled pro-ANF were added to either atrial or ventricular culture wells, no significant cleavage was observed despite normal cleavage of endogenously produced ANF (data not shown). Also, addition of a cocktail of protease inhibitors to the secretion medium (µg/mL: PMSF, 1.7; leupeptin, 1; bacitracin, 1; and
TABLE 4. Effect of Brefeldin A and Inhibitors of Signaling on Atrial Natriuretic Factor Secretion From Atrial Myocytes Maintained in Culture for 60 Hours

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ANF Secretion, pg total ANF (well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td>5 µg/mL Brefeldin</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>3 mmol/L EGTA</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>50 µmol/L Pinacidil</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>100 nmol/L Staurosporine</td>
<td>0.04±0.01*</td>
</tr>
<tr>
<td>3 mmol/L EGTA + 50 µmol/L pinacidil + 100 nmol/L staurosporine</td>
<td>0.03±0.01*</td>
</tr>
</tbody>
</table>

ANF indicates atrial natriuretic factor. Values are mean±SEM (n=4 independent studies per treatment). ANF secretion is presented as picograms ANF released into the medium per picograms total ANF per well.

benzamidine, 1) had no effect on ANF processing (82±2% with protease inhibitors versus 84±4% in control atrial cells; 69±5% with inhibitors versus 70±7% in control ventricular cells; n=2). These findings are in agreement with those of Sei et al.,15 who found no evidence to support the hypothesis that processing occurs by cells other than the myocytes themselves.

The Effect of Time in Culture on the Atrial Secretory Pathway

Neonatal atrial myocytes undergo changes in culture over time. This is predominantly due to a redifferentiation process that occurs after cell dispersion with trypsin and reattachment to the culture dish, which is somewhat dependent on the composition of the culture media. At up to 5 days in culture, ANF release from atrial cells cannot be stimulated by secretagogues such as KCl or endothelin, and total cellular ANF content is low.17,18 Thus, it is possible that a switch from the constitutive to regulated secretory pathway occurs as atrial cells redifferentiate. The existence of this mechanism was explored using brefeldin A.

After 60 hours in culture, atrial myocytes were observed to be morphologically different from cells observed for 2 to 3 weeks. At this “early” time, myocytes had attached to the dish and resembled spindles but were visible as individual quiescent cells. In addition, endothelin had no effect on ANF secretion from atrial myocytes (ANF release was 103% of the control value), and ANF processing to the active form was minimal [11±2% of released ANF was in the ANF-(99–126) form, n=2]. These results confirm earlier observations by Shields et al10 and Sei and Glembotski.17 However, as demonstrated in Table 4, brefeldin A did not inhibit cellular secretion, indicating no constitutive secretion of ANF. In these nonbeating cells, the predominant mechanism of basal ANF secretion is apparently protein kinase C-mediated, since neither cell hyperpolarization by pinacidil nor chelation of extracellular Ca2+ by EGTA significantly inhibited secretion (Table 4). This finding contrasts with the results obtained with atrial myocytes maintained in culture for 3 weeks (Table 2) and may represent another time-dependent difference in cell function. Thus, these findings indicate that the regulated secretory pathway is maintained in atrial myocytes even during a developmental period characterized by reduced Ca2+ channel and sarcoplasmic reticulum activity,27,28 reduced responses to secretagogues (perhaps due to receptor-effector uncoupling),18 and an absence of ANF processing activity.

Discussion

Brefeldin A clearly defines the cell-specific pathways of ANF secretion that exist between neonatal atrial and ventricular myocytes. Neonatal ventricular ANF secretion is entirely dependent on the availability of newly synthesized hormone, whereas atrial ANF secretion is unaffected by brefeldin A blockade of protein transport. Consistent with these findings, ventricular cell ANF secretion is insensitive to signaling inhibitors that decrease atrial ANF secretion. These results suggest that ventricular ANF is secreted via the constitutive secretory pathway, whereas atrial ANF secretion is mediated via a regulated pathway. Under basal conditions, there is no evidence for the existence of dual pathways of ANF secretion in either atrial or ventricular cells, even at early, less differentiated stages in culture.

During the course of the present study, others have confirmed that neonatal ventricular cells possess no dense-cored storage granules and release newly synthesized ANF rapidly, yet they have also observed that endothelin transiently stimulates ventricular secretion.9 We have been unable to demonstrate endothelin-stimulated ANF secretion from ventricular cells, suggesting that it may depend on experimental conditions. If confirmed, the observation may lead to the identification of a novel secretory pathway that is perhaps unique to neonatal ventricular myocytes: regulated release of “constitutive” secretory vesicles.

The enhancement of regulated secretion in atrial myocytes by brefeldin A was a surprising finding. Although this has also been observed in isolated atria,21 it has not been reported with the use of brefeldin A in other endocrine tissues. In fact, brefeldin A inhibits the formation of immature secretory granules at the trans-Golgi network.29,30 It is well established that brefeldin A has multiple effects on vesicular transport. Effects at the level of the Golgi apparatus are thought to be mediated by loss of β-COP, a coat protein complex believed to be important in the segregation of intracellular membranes. Changes in the association of coat proteins with membranes may also be responsible for the inhibition of transcytotic vesicle formation observed with brefeldin A.13,14 However, it is difficult to envision how this mechanism could specifically increase only regulated ANF secretion. Another possibility is suggested by the work of Zizi et al.31 They have reported that brefeldin A acts as an ionophore, increasing K+ and Na+ transport when added to planar lipid bilayers at a concentration of 1 µg/mL. This may increase the osmotic stress on granule membranes, making them more prone to lysis. Increased Na+ influx may also directly stimulate secretion by depolarizing the cells or may increase cellular [Ca2+] via Na+-Ca2+ exchange.
The brefeldin A–induced increase in atrial ANF secretion complicated determination of whether there was a small constitutive component to atrial ANF secretion. It would be possible that a decrease in constitutive atrial secretion could be masked by an increase in regulated secretion. Thus, the results of adding signaling inhibitors (Table 2) were important to show that atrial secretion is primarily a regulated process. The inhibition of atrial secretion by agents that affect cell depolarization, Ca\(^{2+}\) influx, and protein kinase C support the idea that atrial release of ANF should be continuous from a regulated pathway because of the cyclic generation of second-messenger signals in a beating heart. This idea is supported by a recent study demonstrating that rhythmic exocytosis paralleled secretagogue-induced Ca\(^{2+}\) oscillations in single cells of rat gonadotropes.32

An additional insight from the signaling study is the role of protein kinase C in basal ANF secretion from both long-term (21-day) and short-term (3-day) cultures of neonatal myocytes. This suggests that protein kinase C may be activated to some extent in normal beating cells. The data in Table 2 also indicate a possible interaction between the Ca\(^{2+}\) and protein kinase C signaling pathways in the regulation of ANF secretion. Inhibition of both of these pathways does not have an additive effect as might be expected. A permissive level of protein kinase C activity may be required to stimulate secretion via the Ca\(^{2+}\)-dependent pathway, or increased [Ca\(^{2+}\)] may further stimulate phospholipase C and augment protein kinase C activity. The responses observed with the antagonists of signaling are consistent with the synergistic increase in ANF secretion observed when A23187 (a Ca\(^{2+}\) ionophore) and TPA (a protein kinase C activator) are added together.3,33

Table 2 also indicates that there is a role for intracellular Ca\(^{2+}\) stores in atrial ANF secretion. Iida and Page34 showed no inhibition of secretion by ryanodine and have disputed the role of intracellular Ca\(^{2+}\) stores in ANF secretion. However, the myocytes used in their experiments were cultured adult cells that may have altered sarcoplasmic reticulum Ca\(^{2+}\)-loading properties.35 In keeping with our data, Sei and Glembocki17 reported that ionomycin depletion of Ca\(^{2+}\) stores inhibits ANF secretion. It is likely that the latter results are physiologically correct, since much of the cytosolic [Ca\(^{2+}\)] mobilized in cardiac cells is from intracellular stores56 and Ca\(^{2+}\) influx is tightly coupled to intracellular release.24 Unlike neural or endocrine cells, Ca\(^{2+}\) release from cardiac Ca\(^{2+}\) stores has been shown to be due to Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Thus, release from Ca\(^{2+}\) stores does not occur in the absence of Ca\(^{2+}\) influx, and Ca\(^{2+}\) influx normally stimulates Ca\(^{2+}\) release from the sarcoplasmic reticulum.

Brefeldin A was also used to determine whether pro-ANF processing was correlated with the regulated secretory pathway, as it is in many endocrine cell types. The lack of correlation found is unusual, although pro-ANF processing to ANF-(99–126) in the heart has been shown to be unique in several ways. First, ANF in cardiac cells is stored as the prohormone, whereas granular processing of pro-ANF occurs in extracardiac tissues.37,38 Processing by atrial cells is believed to occur as ANF is secreted or in “transient processing vesicles.”18 Second, the small quantities of ANF found in other tissues are cleaved at different sites in the molecule compared with the heart.39 Based on the results obtained in this study, a remaining issue is how and where processing occurs. The two major possibilities are that there is one enzyme cleaving pro-ANF in both atrial and ventricular cells or that separate enzymes with common substrate specificity are associated with different secretory pathways. If there is a single enzyme, it may be localized near the plasma membrane in both cell types or may be capable of functioning in association with either regulated or constitutive secretory vesicles. The dependence of processing activity on the presence of glucocorticoids in both tissues is consistent with the hypothesis of a single enzyme. Alternatively, there may be atrium- and ventricle-specific pro-ANF–processing enzymes forms analogous to, but not identical with, the newly discovered prohormone convertases (PC2 and PC3) and furin.40 These endoproteases all cleave at basic residues and can yield similar products, yet the prohormone convertases PC2 and PC3 are expressed exclusively in tissues with regulated pathways, whereas furin is widely expressed among many tissues and is likely associated with constitutive secretion.

The observation that ventricular cells process pro-ANF is of interest in interpreting data on ANF secretion from hypertrophied ventricle. Although normal adult ventricle expresses little ANF, the ventricle reverts to a more fetal phenotype during hypertrophy or congestive heart failure, and ANF biosynthesis and secretion resumes.41 Additionally, there is a measurable increase in circulating pro-ANF that is physiologically inactive.42 It was previously supposed that the increase in unprocessed ANF was due to the increase in constitutive secretion from ventricle; this would be reasonable if the processing enzyme could function only in the secretory granule environment. However, we have demonstrated that the enzyme is active in association with the constitutive secretory pathway as well. Therefore, it is likely that the reduction in ANF processing found in patients with congestive heart failure is not simply a consequence of an increase in the constitutive release of ANF from the ventricle. Rather, it is most likely due either to a defect in the production or activity of the processing enzyme or to the release of ANF from damaged cells.

In summary, the present study confirms and further delineates several important characteristics of the cardiac ANF secretory system. First, neonatal atria and ventricles use different secretory mechanisms despite expression of the ANF gene by both cell types. No regulated ANF secretion from ventricle was found, whereas atrial ANF secretion has no apparent constitutive component, even at early times in culture. Second, basal ANF secretion from the atria is regulated, in part, by the second-messenger signals found to be active in most neural or endocrine cells (Ca\(^{2+}\) depolarization and protein kinase C), although in myocytes these signals may be associated with stretch activation. Cardiac differences in the expression of Ca\(^{2+}\) channels, Ca\(^{2+}\)-ATPases, and Ca\(^{2+}\) storage mechanisms seem to make little difference to the exocytotic machinery. Thus, the basal, or spontaneous, secretion observed in these in vitro experiments most likely reflects continuous release...
of ANF from a beating heart in vivo. Third, pro-ANF processing activity is observed whether pro-ANF is secreted from a constitutive or regulated pathway and may be impared with hypertrophy.

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