Phasic Secretion of Newly Synthesized Atrial Natriuretic Factor From Unstimulated Atrial Myocytes in Culture

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Abstract We have examined kinetics and composition of newly synthesized proteins secreted from cultured atrial myocytes from adult rats. Under unstimulated conditions, noncontracting cultured atrial myocytes, which were pulse-labeled for 10 minutes with [35S]methionine, rapidly released a considerable portion of newly synthesized atrial natriuretic factor (ANF) in a phasic secretion with a peak at 40 to 80 minutes of chase time. The phasic secretion almost ceased after 80 minutes of chase, after which relatively slow release of the hormone was observed. The ability to stimulate the phasic secretion with secretagogues and a marked resemblance of the radiochemical composition of released proteins in the unstimulated phasic secretion to that in stimulated secretion suggest that the proteins discharged from the cells during the phasic secretion might be derived from secretory granules. Examination of the quantitative change of intracellular ANF showed that ≈60% of newly synthesized labeled ANF was still retained in the cells after the termination of the phasic secretion, indicating that the termination of the phasic secretion was not due to depletion of the labeled protein in the cells. These results suggest that a proportion of newly synthesized ANF was rapidly released from the unstimulated atrial myocytes via a secretory route that shares certain features with both the regulated and the constitutive secretory pathway and that a part of newly synthesized ANF is processed for rapid release while the remainder is destined for slow release or storage within the cells. (Circ Res. 1994;74:659-668.)

Key Words • atrial natriuretic factor • atrial myocytes • secretion • regulated pathway • constitutive pathway

Secretory proteins in eukaryotic cells can follow either an intracellular route that leads directly to the cell surface (constitutive pathway) or a route that leads to specialized secretory granules that can store the proteins in a highly concentrated form (regulated pathway).1,2 The concentrated contents in the secretory granules are released from cells in response to external signals, whereas no signals are required for the constitutive secretion.1,2 The sorting of secretory proteins into the regulated or the constitutive pathways takes place in the reticular trans-Golgi network (TGN),1,3 which might be caused either by receptor-mediated protein targeting4 or by intraluminal protein aggregation and condensation.5 Since it has been shown that markers for the constitutive and the regulated pathways were found to be colocalized in the TGN but that only the regulated secretory proteins were detected in secretory granules,6,7 it has been generally assumed that the sorting of secretory proteins at the TGN is efficient. This view was supported by the results reported in some types of cells, such as pancreas B cells, in which 99% of newly synthesized insulin/proinsulin is released through the regulated pathway and the contribution of the constitutive pathway is very small, accounting for only 1% of the hormones released from the B cells.8 In some types of cells, however, it has been postulated that regulated proteins that fail to be properly sorted into the regulated pathway are released from cells in the absence of stimulation: 15% of the newly synthesized secretory proteins exit through the constitutive pathway in the exocrine pancreas,9 and lactating mammary cells seem to use both pathways to secrete caseins.10 Thus, the sorting efficiency of regulated secretory proteins into the regulated pathway seems to vary between secretory cell types.

Atrial natriuretic polypeptide hormone (atrial natriuretic factor [ANF]), which is an important regulator of circulating blood volume and of vascular smooth muscle functions,11 is released from atrial myocytes. To investigate the mechanisms by which ANF is released from the atrial myocytes, we have previously measured the rate of ANF secretion by radioimmunoassay using primary culture of the atrial myocytes from adult rats.12-14 These studies showed that, although the cells increased the rate of ANF secretion in response to stimulation with secretagogues, the cells also secreted ANF into the culture media in the absence of secretagogues even when their spontaneous contraction was inhibited. Since this unstimulated release of ANF, which was also observed in cultured atrial myocytes from neonatal rats15 as well as in isolated intact rat atria,16,17 accounts for a considerable portion of ANF secreted from the cells, it might have important implications for the analysis and the understanding of ANF sorting and secretion.

In the present study, we have examined the kinetics and radiochemical composition of [35S]-labeled secretory proteins released from the cultured atrial myocytes to elucidate whether the unstimulated release of the secretory proteins is caused either by inefficient sorting of the proteins, which might result in targeting them into
the constitutive pathway, or by other mechanisms, such as spontaneous fusion between the plasma membranes and secretory granules in the absence of stimulation.

**Materials and Methods**

**Cell Culture**

Primary cultures of atrial myocytes were prepared from the hearts of ether-anesthetized 200- to 250-g Wistar rats as previously described. 12-14 The cells were cultured in laminin-coated 35-mm dishes at 37°C with medium 199 (M199) supplemented with 10% fetal bovine serum in an incubator. The cells were used for experiments on day 7 or day 8 of culture. Cultured atrial myocytes, which had spread fully over the laminin-coated substrate and showed spontaneous contraction, often extended several cell processes, by which they made contact with other myocytes as described previously. 12 The cells had well-developed contractile filaments that were observed after staining with fluorescein isothiocyanate–conjugated phalloidin (not shown).

**Biosynthetic Labeling**

For pulse-chase experiments, cells were labeled for 10 minutes by incubation with 170 μCi/mL [35S]methionine (Amersham Corp) in methionine-depleted M199 culture medium containing 10% dialyzed fetal bovine serum. After washing with M199 containing excess cold methionine (1.5 mg/mL), cells were incubated in 1 mL of M199 containing cold methionine and 100 μmol/L ryanodine for successive 20-minute intervals. At the end of the chase intervals, duplicate 400-μL samples of culture medium containing secreted proteins were mixed with 500 μL of 20% trichloracetic acid (TCA) and 100 μL of 1% bovine serum albumin as a carrier, kept on ice for 30 minutes, and centrifuged at 4°C for 5 minutes at 10,000 rpm to collect precipitated proteins. The pellets were washed twice with 800 μL of 10% TCA and collected by centrifugation, and then precipitated proteins were dissolved either in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample buffer for electrophoresis or in Aquasol II (NEN Research Products, Boston, Mass) for analysis by liquid scintillation counting. In some cases, cells were labeled and chased for various times as specified in the text. Secreted labeled proteins were collected by TCA precipitation as described above. For analysis of intracellular labeled proteins, cell extracts were prepared by solubilizing cells in a dish in 1 mL solubilizing buffer (10 mmol/L Tris, pH 7.4, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, and 2 mmol/L EDTA) containing phenylmethylsulfonyl fluoride (0.5 mmol/L), aprotinin (1 μU/mL), and leupeptin (0.1 mmol/L). After centrifugation to remove undissolved materials, labeled proteins in the samples were precipitated by TCA as described above. For biosynthetic labeling with cysteine, the cultured cells were labeled with 340 μCi/mL of [35S]cysteine (Amersham) and proteins in the medium, and cells were processed and analyzed as described for the labeling with [35S]methionine. For continuous labeling, the cells were labeled for various times by incubation with 25 μCi/mL [35S]methionine in methionine-depleted M199 in the presence of 100 μmol/L ryanodine. After labeling, secreted proteins in the culture medium were precipitated by TCA for liquid scintillation counting or SDS-PAGE and fluorography as described in the pulse-chase experiments.

In some cases, secretion of proteins from the atrial myocytes was either stimulated by incubation of the cells with 100 nmol/L endothelin 1 (ET, Peptide Institute Inc, Osaka, Japan), 100 nmol/L 12-O-tetradecanoylphorbol 13-acetate (TPA, Sigma Chemical Co, St Louis, Mo), or 50 mmol/L KCl or suppressed by incubation with 5 mmol/L EGTA and BAPTA-AM (Molecular Probes, Eugene, Ore). TPA and BAPTA-AM were dissolved in dimethyl sulfoxide (DMSO), The final concentration of DMSO was 0.006% for TPA and 0.03% for BAPTA-AM. The control cells were incubated with the medium containing only DMSO. A protein kinase C inhibitor, 1-(S-isooquinolinylsulfonfonyl)-2-methylpiperazine dihydrochloride (H-7), and a Ca2+/calmodulin kinase inhibitor, KN-62, both of which were obtained from Seikagaku Kogyo, Tokyo, Japan, were used to examine the effects of these kinase inhibitors on protein secretion from the cells.

**Immunoprecipitation**

Samples for immunoprecipitation were prepared by mixing 400 μL of culture media and 400 μL of 2× solubilizing buffer containing the protease inhibitors. Cell extracts (800 μL) prepared in solubilizing buffer described above were directly used for immunoprecipitation. Samples were precleared by incubation for 30 minutes at room temperature with pre-washed protein-A agarose (Boehringer Mannheim). The materials that nonspecifically bound to the agarose were removed by centrifugation, and the supernatants were incubated for 16 hours at 4°C with antisera either to α-ANF (C-terminal 99-126 of pro-ANF, UCB Bioproducts, Brussels, Belgium) or to pro-ANF (Peninsula Laboratories, Belmont, Calif). After incubation with protein-A agarose for 2 hours at room temperature followed by centrifugation, the supernatants were incubated a second time with another aliquot of the fresh antibodies and protein-A agarose, which resulted in no further detectable labeled proteins in the immunoprecipitates. Immunoprecipitated proteins were dissolved in SDS-PAGE sample buffer and fractionated on slab gels either for fluorography or for quantification by liquid scintillation counting of excised gels containing ANF-related polypeptides. For the control condition, samples were incubated with preimmune rabbit serum followed by incubation with protein-A agarose. We could not detect any precipitated proteins in the samples for the controls by SDS-PAGE and fluorography.

**SDS-PAGE and Fluorography**

Sample proteins that were either precipitated by TCA or immunoprecipitated by antiserum to ANF were dissolved in SDS-PAGE sample buffer and separated on polyacrylamide gels. For fluorography, the gels were fixed, soaked in Amplify (Amersham), vacuum-dried onto filter papers, and exposed at –70°C to Hyperfilm MP (Amersham). The amounts of labeled 17-kD pro-ANF and 14-kD N-terminal of pro-ANF were quantified by cutting out the parts of the gels containing the polypeptides, eluting the labeled proteins in Solvable (NEN Research Products) at 50°C for 3 hours, and counting in a liquid scintillation counter in scintillation vials containing Atomlight (NEN Research Products).

**Electron Microscopy**

Cultured cells were prepared for electron microscopy as described previously, 12 and thin sections were examined in a JEOL 2000EX electron microscope.

**Results**

**Molecular Form of ANF Secreted From Atrial Myocytes in Culture**

To investigate the nature of the proteins synthesized and secreted from cultured atrial myocytes from adult rats, the cells were incubated for 60 minutes with [35S]methionine, which would be incorporated into the N-terminal region of pro-ANF (Fig 1), and labeled proteins in both the cells and the culture medium were collected either by TCA precipitation or by immunoprecipitation. Fluorography of precipitated proteins separated on SDS-PAGE shows that, of a number of labeled proteins incorporated into the cells, a limited number of polypeptides corresponding to 14-, 17-, and 40-
The 17-kD polypeptide in the cells was selectively immunoprecipitated by antisera to α-ANF, indicating that the polypeptide is the storage form of ANF, i.e., pro-ANF (Fig 2a). The incubation medium contained 14- and 17-kD polypeptides, both of which were immunoprecipitated by antisera to pro-ANF (Fig 2b), whereas only the 17-kD polypeptide (pro-ANF) was immunoprecipitated by antisera to α-ANF (Fig 2a). Since methionine only occurs in the N-terminal region of pro-ANF (Fig 1), 3-kD ANF, a cleaved product of pro-ANF, would not be labeled by [35S]methionine. These results show that 17-kD pro-ANF is the storage form of ANF in the cells, whereas the culture medium bathing the cells labeled with [35S]methionine contains two major secreted proteins, the 17- and the 14-kD polypeptides, which should correspond to pro-ANF and the N-terminal of pro-ANF, respectively.

Similar experiments were done using [35S]cysteine, which would be incorporated into the C-terminal region of pro-ANF (Fig 1). SDS-PAGE followed by fluorography showed that 17-kD pro-ANF in the cells and both 17-kD pro-ANF and the 3-kD polypeptide in the medium were immunoprecipitated by antisera to α-ANF (Fig 2c). The 3-kD polypeptide should correspond to the C-terminal of pro-ANF (α-ANF) (Fig 1). Since cysteine only occurs in the C-terminal region of pro-ANF (Fig 1), the 14-kD N-terminal of pro-ANF would not be labeled by the amino acid. Thus, the culture medium bathing the cells labeled with [35S]cysteine contains both [35S]-labeled 17-kD pro-ANF and 3-kD ANF. In addition to these ANF-related peptides, the cells labeled with [35S]cysteine secreted immuno-non-precipitable 45-kD protein into the medium (Fig 2C).

The data shown here are consistent with the chromatographic data of Suzuki et al., who showed that pro-ANF (pro-ANF), the storage form of ANF in the cells, is cleaved into an N-terminal region and a C-terminal region (i.e., α-ANF) after secretion in cultured atrial myocytes from neonatal rats.

Continuous Labeling Experiments

To assess the secretory activity of the cultured atrial myocytes in the absence of stimulation, the cells were labeled for various times with [35S]methionine in methionine-depleted medium containing 100 μmol/L ryanodine, which was added to the medium to inhibit spontaneous contraction of the cells. After incubations of 15, 30, 60, 90, and 120 minutes, secreted proteins in the medium were collected by TCA precipitation for analysis either by liquid scintillation counting (Fig 3A) or by SDS-PAGE and fluorography (Fig 3B). Secretory kinetics of ANF (17-kD pro-ANF plus 14-kD N-terminal of pro-ANF) were determined by densitometric quantification of the data in Fig 3B using a National Institutes of Health Image System (Fig 3C). The results showed that, in the absence of secretagogues, secretion of both total proteins (Fig 3A) and ANF (Fig 3C) was almost linear with time, except for the initial stage of secretion.

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**Figure 2.** Characterization of proteins synthesized and secreted by cultured atrial myocytes labeled with [35S]amino acids. Atrial myocytes were labeled for 60 minutes with 100 μCi/mL of [35S]methionine (a and b) or [35S]cysteine (c), and labeled proteins were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography. a. Labeled proteins in the cells (C) and medium (M) were recovered either by trichloroacetic acid (TCA) precipitation (column A) or by immunoprecipitation using antisera to α-atrial natriuretic factor (α-ANF) (column B). Seventeen-kilodalton pro-ANF (indicated by arrow) was selectively immunoprecipitated, whereas 14-kD polypeptide in the medium and proteins positioned at 40 to 45 kDa were not immunoprecipitated by the antibody. b. Seventeen-kilodalton pro-ANF and 14-kD N-terminal of pro-ANF in the medium were immunoprecipitated by antisera to pro-ANFα92 (lane 1). Preimmune serum did not precipitate any proteins (lane 2). An arrow indicates 17-kD pro-ANF. c. Labeled proteins in cells (C) and medium (M) were recovered by TCA precipitation (column A) or by immunoprecipitation using anti-α-ANF antibody (column B). Both 17-kD pro-ANF (indicated by arrow) and 3-kD α-ANF (indicated by double arrows) in the medium were immunoprecipitated.
incubation between 0 and 30 minutes, in which a relatively slow release of proteins was observed.

**Pulse-Chase Experiments**

To understand the cellular basis for unstimulated release of ANF from the cultured atrial myocytes (Fig 3), we have designed the experiments examining secretion during noncumulative chase incubations after a 10-minute pulse labeling with [35S]methionine. In the presence of 100 μmol/L ryanodine and in the absence of secretagogues, a phasic secretion of newly synthesized proteins with a broad peak at 40 to 80 minutes of chase time was observed by liquid scintillation counting of TCA-precipitated radiolabeled proteins (Fig 4A). The phasic secretion almost ceased 80 minutes after pulse labeling, after which relatively slow release of labeled proteins was observed for up to 160 minutes of chase (Fig 4A). Stimulation of the cells with TPA at delayed chase time (160 minutes after pulse labeling) resulted in increased discharge of labeled proteins, suggesting the presence of labeled proteins in the secretory granules (Fig 4A). Analysis of the nature of the TCA-precipitated proteins by SDS-PAGE and fluorography indicated that, in the absence of stimulation, the cells secreted almost the same proteins throughout the chase period, with major polypeptides corresponding to the bands at 17 and 14 kD and that these two polypeptides were released with a broad peak at 40 to 80 minutes of chase time (Fig 4B). Immunoprecipitation experiments confirmed that the 17- and the 14-kD proteins were pro-ANF and the N-terminal of pro-ANF, respectively (not shown), as shown in Fig 2. Several polypeptides corresponding to the bands at 40 to 46 kD were also detectable throughout the chase period (Fig 4B). Fig 4B also shows that the radiochemical composition of proteins (ie, species of labeled proteins) released in the presence of TPA was not qualitatively different from that released in the absence of the secretagogue. The secretory kinetics of the ANF-related proteins were examined in more detail by liquid scintillation counting of excised gels containing both 17- and 14-kD bands. The results confirmed the phasic secretion of the proteins at 40 to 80 minutes of chase time (Fig 4C). The phasic secretion of labeled proteins could not be ascribed to cell injury or cell death, since a limited number of labeled proteins was selectively secreted into the media throughout 3 hours of chase time (Fig 4B). Furthermore, phase-contrast microscopy showed that the cells were intact during the 3 hours of the chase period (not shown).

To determine whether the termination of the phasic secretion of ANF by 80 minutes of incubation (Fig 4) was due to depletion of labeled pro-ANF in the cells, we examined the quantitative change of pro-ANF in the cells that were pulse-labeled for 10 minutes with [35S]methionine followed by chase for up to 160 minutes in the absence of stimulation. Cell lysates were prepared after 0, 20, 40, 60, 80, 100, 120, 140, and 160 minutes of chase in the presence of 100 μmol/L ryanodine and excess cold methionine. Intracellular pro-ANF was recovered by immunoprecipitation, and precipitated pro-ANF was examined either by SDS-PAGE and fluorography (Fig 5A) or by liquid scintillation counting of excised gels containing 17-kD pro-ANF (Fig 5B). The results showed that the incorporation of [35S]methionine into newly synthesized pro-ANF rapidly occurred after a 10-minute pulse labeling peaking at 20 minutes of chase time and that, as the chase time increased, labeled pro-ANF in the cells decreased. After 80 minutes of incubation, at which time point the phasic secretion of ANF almost ceased (Fig 4), the cells still contained ~60% of labeled pro-ANF synthesized by 20 minutes of chase time (Fig 5B), suggesting that the termination of the phasic secretion of ANF may not be ascribed to depletion of newly synthesized pro-ANF in the cells.

We then examined what percentage of newly synthesized ANF is released in the absence of stimulation by quantification of both released ANF in the medium and ANF kept in the cells. Chase in this experiment was started after 20 minutes of incubation following a 10-minute pulse labeling: Fig 5 shows that the amount
of immunoprecipitable ANF that was synthesized in the cells was small after a 10-minute pulse labeling and reached a maximum after 20 minutes of incubation following 10-minute pulse labeling. The cells were pulse-labeled for 10 minutes with [35S]methionine, incubated for 20 minutes in the presence of 100 μmol/L ryanodine, and then chased for 0, 20, 40, 60, 80, 100, 120, and 140 minutes in the presence of 100 μmol/L ryanodine. Both released ANF in the medium and intracellular ANF were recovered by immunoprecipitation at the end of each chase period. Immunoprecipitated proteins were subjected to SDS-PAGE and fluorography (Fig 6A and 6B), and quantification of 17-kD pro-ANF in the cells and both 17-kD pro-ANF and 14-kD N-terminal of pro-ANF in the medium was performed by liquid scintillation counting of excised gels containing these proteins. The result showed that secreted ANF accounted for 37% and 58% of total synthesized ANF after 60 and 140 minutes of chase, respectively (Fig 6C).

Enhanced Release of ANF by Stimulation in the Phasic Secretion

From the data above, it appears that a considerable portion of newly synthesized ANF is rapidly released in a phase of secretion from the noncontracting atrial...
myocytes in the absence of stimulation. We then examined whether the proteins discharged during the phasic secretion were released through a secretagogue-insensitive secretory pathway or the constitutive pathway. After pulse labeling for 10 minutes with [35S]methionine, cells in six dishes were washed and chased in the medium containing 100 μmol/L ryanodine for three 20-minute intervals. During the third interval, at which time the cells should be situated near the peak of the phasic secretion (see Fig 4), the cultured cells were divided into two groups: one for control and the other for stimulation with 100 nmol/L ET and 100 nmol/L TPA. Labeled secreted proteins were precipitated by TCA for liquid scintillation counting or SDS-PAGE followed by fluorography. The results showed that exposure of the cells to the secretagogues during the third chase interval amplified the discharge of labeled proteins 2.2-fold (Fig 7A). Fluorography of secreted proteins fractionated on SDS-polyacrylamide gels showed the secretagogue-enhanced discharge of the 17-kD pro-ANF and the 14-kD N-terminal of pro-ANF, although the radiochemical composition of secreted proteins in the presence of secretagogues was not qualitatively different from that in their absence (Fig 7B). The amounts of ANF-related proteins that were discharged during the third interval in the presence or the absence of secretagogues were quantified by counting excised gel bands containing 17-kD pro-ANF and 14-kD N-terminal of pro-ANF. The results confirmed that the discharge of both proteins was enhanced by stimulation with secretagogues 2.7-fold (Fig 7C). In addition, these observations suggest that a considerable portion of labeled ANF was concentrated and packed into the secretory granules by 40 minutes after pulse labeling.

Radiochemical Composition of Secreted Proteins Is Unaffected by Secretagogues and Inhibitors of Secretion

To examine further whether the radiochemical composition of proteins secreted from the cultured atrial myocytes would be changed by the addition of secretagogues or inhibitors into the culture media, the cells were labeled for 30 minutes with [35S]amino acids, washed, and then incubated for 30 minutes in their presence or absence. Secreted labeled proteins were collected by TCA precipitation for examination by SDS-PAGE and fluorography. A Ca2+ chelator, BAPTA-AM, which was used as an inhibitor of secretion, would be expected to be taken up by the cells and cleaved. The free BAPTA would then chelate cytosolic Ca2+. The chelator was used in combination with 1.5 mmol/L EGTA, which was added to the medium to
reduce the free Ca\(^{2+}\) concentration of the culture medium from 1.5 mmol/L to 45 \(\mu\)mol/L (determined by a Ca\(^{2+}\) electrode). Exposure of the cells to the medium containing EGTA/BAPTA-AM completely abolished cellular contraction.

As shown in Fig 8A, in the absence of ryanodine the radioactive composition of proteins secreted from the cells labeled with \(^{35}\)S methionine was unaffected by stimulation with ET, TPA, and depolarization by 50 mmol/L KCl, although these secretagogues seemed to increase the release of \(^{35}\)S-labeled proteins. Similarly, the radiochemical composition of proteins discharged from ryanodine-treated cells labeled with \(^{35}\)S cysteine did not differ qualitatively either from that of EGTA/BAPTA-AM–treated cells or from that of TPA-treated cells (Fig 8B).

We also examined the effects of H-7 and KN-62 on the radiochemical composition of proteins secreted from the cultured atrial myocytes (Fig 8C). H-7 and KN-62 are potential inhibitors for protein kinase C and Ca\(^{2+}/\)calmodulin kinase, respectively.\(^{19}\) The results showed that the radiochemical composition of proteins...
discharged from control cells did not differ qualitatively either from that of ryanodine-treated cells or from that of cells treated with H-7 and KN-62. Quantification of both 17-kD pro-ANF and 14-kD N-terminal of pro-ANF discharged from ryanodine-treated cells (Fig 8C, lane 2) and inhibitor-treated cells (Fig 8C, lane 3) was performed by liquid scintillation counting of excised gels containing both proteins. The result showed that discharge of both proteins was suppressed by inhibition with the kinase inhibitors by \( \approx 28\% \).

Thus, neither secretagogues nor inhibitors of secretion are able to change the radiochemical composition of proteins secreted from the cells.

**Morphological Study of Secretory Granules in the Cultured Atrial Myocytes**

Arvan and coworkers\(^{20-22}\) proposed a third secretory pathway or the constitutive-like pathway by which regulated secretory proteins that aggregate inefficiently in immature secretory granules are preferentially segregated in clathrin-coated vesicles budding from the immature secretory granules and secreted in the absence of stimulation. They proposed that unstimulated release of regulated proteins from secretory cells might occur through this secretory pathway. Therefore, we examined the secretory granules in the cultured atrial myocytes to search for such vesicles budding from the immature secretory granules, which can be morphologically identified by the presence of clathrin coats on their membranes.\(^{23}\) Electron microscopy showed that clathrinlike coats were actually common in the secretory granules in the perinuclear region of cultured atrial myocytes (Fig 9A and 9B). Eight percent (\( n = 120 \)) of the secretory granules had clathrinlike coats. We also identified, though rarely, secretory granules having a coated protrusion, which appeared to be in the process of budding off to form discrete transport vesicles (Fig 9C).

**Discussion**

Cardiac atrial myocytes contain secretory granules in which an ANF hormone is concentrated. Neonatal ventricular myocytes also synthesize ANF but have few secretory granules.\(^{24}\) Bloch et al\(^{24}\) reported in 1986 that the ratio of secreted ANF to cellular ANF was much higher in cultured ventricular myocytes than in cultured atrial myocytes and that a considerable amount of ANF was secreted rapidly after synthesis of the hormone in the ventricular myocytes than in the atrial myocytes. In addition, secretion of ANF from the atrial myocytes has been shown to be enhanced by stimulation with secretagogues such as ET, phorbol ester, and Ca\(^{2+}\) channel activators.\(^{13,15,19,25-27}\) Therefore, it is generally believed that ANF is secreted from the atrial myocytes through the regulated pathway and that ANF from the ventricular myocyte is secreted via the constitutive pathway.

On the other hand, we have previously observed that, in cultured atrial myocytes from adult rats, a considerable amount of ANF was spontaneously released from the unstimulated atrial myocytes into the culture media and that this unstimulated secretion continued for at least several hours even when cell contraction was suppressed.\(^{13,14}\) Similar results have been recently reported in cultured atrial myocytes from neonatal rats.\(^{15}\) Page and colleagues\(^{16,17}\) also reported that immunoreactive atrial natriuretic peptide (ANF) was secreted from quiescent (noncontracting and unstimulated) intact atria and that this unstimulated release of ANF seemed to occur via a cycloheximide-insensitive secretory pathway that might be distinct from the constitutive pathway. These results suggest that the regulated path-
way, which is defined by the release of secretory proteins by stimulation,\textsuperscript{1,2} cannot account for all aspects of secretory phenomena of ANF observed in atrial myocytes.

There is a possibility that unstimulated release of newly synthesized ANF in the phasic secretion shown in Fig 4 might be due to incomplete condensation of the hormone at the level of the TGN, thereby allowing a portion of the hormone to pass by default into the constitutive pathway, a secretory pathway that does not require any external signal to discharge proteins.\textsuperscript{1,2} This possibility, however, seems to be unlikely because of the following three reasons: (1) If it is assumed that the proteins discharged in the phasic secretion (Fig 4) are transported and released by constitutive vesicles, the secretion should be unaffected by stimulation with secretagogues. This is not the case, since the results shown in the present study indicate that the phasic secretion of the hormone is enhanced by stimulation with secretagogues (Fig 7). (2) A considerable portion of newly synthesized ANF (\textapprox 40\%) was secreted during the phasic secretion (Fig 6), which cannot be explained solely by missorting of the hormone due to the default at the level of the TGN. (3) Our observation that the radiochemical composition of released proteins during the phasic secretion cannot be distinguished from that of the regulated secretion (Figs 4B, 7B, and 8) suggests that the vesicles responsible for protein discharge in the phasic secretion might be identical with the secretory granules. However, we cannot totally exclude the possibility that unstimulated release of ANF in the phasic secretion is due to the constitutive secretory pathway, because it could be possible that the cultured atrial myocytes have constitutive vesicles that contain the same proteins as those in the secretory granules.

Taken together, these data suggest that a considerable portion of newly synthesized ANF is secreted from the cultured atrial myocytes by a secretory route that shares certain features with both the regulated and the constitutive pathways but is not functionally defined by either one. The ability to stimulate the phasic secretion of ANF with secretagogues and a remarkable resemblance of the radiochemical composition of released proteins in the unstimulated phasic secretion with that in the stimulated secretion suggest that the phasic secretion of the hormone may take place by fusion of the secretory granules with the plasma membranes in the absence of stimulation.

Alternatively, it is also likely that unstimulated secretion of ANF is due to discharge of the contents of small vesicles budded off from immature secretory granules as proposed by Arvan and coworkers,\textsuperscript{20-22} but it is not known whether a coated protrusion with secretory granules (Fig 9C) reflects either the process of budding off, condensing with larger granules, or remodeling of maturing granules.

Another interesting finding emerging from the present study is the observation that the phasic secretion of newly synthesized ANF was found to cease after 80 minutes of incubation (Fig 4), even though \textapprox 60\% of ANF, which was either thereafter released slowly from the cells or stored in the cells, still remained in the cells at that time of the chase period (Figs 5 and 6). These observations suggest that the termination of the phasic secretion of newly synthesized ANF might not be due to depletion of the \textsuperscript{35}S-labeled hormone in the cells. The findings also suggest that a part of newly synthesized ANF is processed for rapid release in a phasic manner while the remainder is destined for slow release or storage within the cells. However, it remains to be determined whether the cultured atrial myocytes have two pools of secretory granules, one for rapid release and the other for slow release. The rapid release and the subsequent slow release of newly synthesized ANF, as well as its synthesis, seem to be elaborately controlled by the cultured atrial myocytes, since radioimmunoassays showed that the cells invariably secreted ANF into culture media at an extremely constant rate in the absence of secretagogue.\textsuperscript{12-14} It is still unknown how synthesis and secretion of ANF are regulated in the atrial myocytes.

The observations that EGTA/BAPTA-AM and protein kinase inhibitors partially suppressed unstimulated release of newly synthesized ANF but failed to inhibit it fully (Fig 8) suggest that unstimulated release of ANF, at least in part, might be Ca\textsuperscript{2+}-independent and protein kinase–independent phenomena. Therefore, it is likely that unstimulated secretion of ANF might reflect a “stimulated” basal state of the atrial myocytes, which could be an inherent characteristic of the cells.

The analysis of secreted proteins by SDS-PAGE and fluorography showed that, in addition to ANF, 40- to 46-kD polypeptides, which could not be immunoprecipitated by antisera to ANF, were released from the cell and that secretion of these proteins seemed to be enhanced by stimulation with secretagogues (Figs 4B, 7B, and 8). These observations suggest that these proteins and ANF might be colocalized in the atrial secretory granules and secreted by fusion between the plasma membranes and the secretory granules. Since these proteins are different in their molecular weights from a peptidase,\textsuperscript{28} atrioactivase,\textsuperscript{29} cathepsin B,\textsuperscript{30} and chromogranins,\textsuperscript{31} all of which have been shown to be localized in the atrial granules, the nature of these proteins remains to be determined.

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