Acid Prohormone Sequence Determines Size, Shape, and Docking of Secretory Vesicles in Atrial Myocytes

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Abstract—How vesicles are born in the trans-Golgi network and reach their docking sites at the plasma membrane is still largely unknown and is investigated in the present study on live, primary cultured atrial cardiomyocytes. Secretory vesicles (n=422) are visualized by expressing fusion proteins of proatrial natriuretic peptide (proANP) and green fluorescent protein. Myocytes expressing fusion proteins with intact proANP display two populations of fluorescent vesicles with apparent diameters of 120 and 175 nm, moving at a top velocity of 0.3 μm/s. The number of docked vesicles is significantly correlated with the number of mobile vesicles (r=0.71, P<0.0005). The deletion of the acidic N-terminal proANP[1-44] or point mutations (glu 23,24→gln 23,24) change size and shape—but not velocity—of the vesicles, and, strikingly, abolish their docking at the plasma membrane. The shapes thus change from spheres to larger, irregular floppy bags or vesicle trains. Deletion of the C-terminal proANP[45-127], where the ANP and its disulfide bond reside, does not change size, shape, docking, or velocity of the mobile vesicles. The N-terminal acid calcium-binding sequence of proANP is known to cause protein aggregation at the high calcium concentration prevailing in the trans-Golgi network. Therefore, these results indicate that amino acid residues favoring cargo aggregation are critically important in shaping the secretory vesicles and determining their fate—docking or not docking—at the plasma membrane. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001;89:e23-e29.)

Key Words: atrium ■ enhanced green fluorescent protein ■ in vivo imaging ■ proatrial natriuretic peptide ■ secretory vesicles

Atrial natriuretic peptide (ANP) is a diuretic vasorelaxant hormone stored in specific secretory vesicles1,2 and is secreted from the heart atria in response to overload and oxygen deficiency.3 Electron microscopy indicates that proatrial natriuretic peptide (proANP) is condensed in the trans-Golgi network2 and because proANP is cleaved only on release,3 secretory vesicles budding off the trans-Golgi network are already mature.4 In contrast, proinsulin must be cleaved to insulin during vesicle maturation before condensation efficiently occurs, and cleavage products are sorted out of the so-called immature vesicles.6 Calcium-dependent condensation of prohormone has been shown for proANP7 and the progranins.8 Mutations of the acid N-terminal of proANP that abolish calcium binding also abolish proANP secretion from transfected AtT-20 cells.9 The defect in secretion was ascribed to a lack of vesicle genesis, although the absence of vesicles has not yet been verified. In the case of the progranins, an N-terminal disulfide-bonded loop was shown to be essential for calcium-dependent aggregation and sorting to regulated secretory vesicles in PC12 cells.8 Interestingly, an acidic amino acid sequence of N-terminal proopiomelanocortin, stabilized by a disulfide bond, is essential for observing a punctate, granule-like staining pattern in transfected Neuro-2a cells,10 although aggregating properties have not yet been identified.

How do the vesicles form? Surface motifs of the prohormone aggregate may recruit lipids and proteins within the membrane of the trans-Golgi network, thereby enwrapping the aggregate and reducing the energy required for pinching off the secretory vesicle budding off the trans-Golgi network.8,11–15 This attractive concept implies that cargo signals determine size and possibly shape of secretory vesicles, but direct evidence is lacking in the tumor cell lines used in those studies, and there is no study at all on live cardiomyocytes. Furthermore, it is unknown whether cargo signals may recruit transmembrane molecules essential for vesicle docking or fusion, although there is evidence that vesicle-associated transmembrane proteins (v-SNARES) will dock the vesicle at a subplasmalemmal protein scaffold.16–18 The present study addresses these questions on primary cultured atrial myocytes, by visualizing fusion proteins of mutated proANP with enhanced green fluorescent protein (EGFP) within moving and docked secretory vesicles. Although atrial myocytes present several technical difficulties, they have the advantage...
of providing a well-differentiated phenotype with mature secretory vesicles carrying uncleaved prohormone to the docking sites. This study identifies the calcium-binding amino acid sequence in proANP as a crucial cargo signal for shaping spherical secretory vesicles of well-defined size. The study also shows that a change in shape to larger floppy vesicles associates with failure of docking at the plasma membrane.

Materials and Methods

Primary Culture of Atrial and Ventricular Myocytes

Atrial cells from neonatal rats (2- to 4-day-old; Sprague-Dawley) were cultured as described previously using enzymatic dispersion (0.2% trypsin; Worthington) of atrial appendages. These cells have distinct ANP-positive microvesicles (Figure 1a), and perinuclear structures closely associated with TGN-38, a marker of the trans-Golgi network (Figure 1d). Ventricular myocytes were cultured by mincing the tips of the left ventricle from 2- to 3-day-old rats into 1- to 2-mm³ fragments and harvesting the cells as described by Sadoshima et al. Cultures were performed weekly by platting 10 000 to 50 000 cells on fibronectin-coated 22-mm glass slides. Between 10% to 50% of the cells examined were contracting.

HL-1 Cell Line

This mouse atrial tumor cell line was reconstituted, split, cultured, and frozen exactly as described in the detailed protocol entitled Care and Feeding of HL-1 Cells (W.C. Claycomb, personal communication, 1998). For Western blots, the cells were plated at medium density and cultured for 2 days in 6-well fibronectin-coated culture plates. For imaging, cells were seeded at low density and cultured for 1 to 2 days on 22-mm fibronectin-coated glass slides. HL-1 cells were transfected with TransFast reagent (Promega).

Plasmid Constructs

Figure 1 (top left) shows a schematic representation of the different fusion proteins of this study. All the plasmid constructs were verified by restriction analysis and DNA sequencing in the coding regions. The pcDNA3-EGFP expression vector was obtained by cloning the full-length EGFP cDNA (pEGFP-1, Clontech) into the pcDNA3 vector from Invitrogen Inc. The pcDNA3-flag-EGFP vector was kindly provided by Philippe Halban (Louis Jeantet Laboratory, Geneva, Switzerland). It is derived from pcDNA3-EGFP containing a sequence flag coding for 9 amino acids (MDYKDDDDK) in frame with the EGFP initiation site. The pcDNA3-signal peptide-proANP[1-41]-flag-EGFP expression vector encodes a chimeric protein composed of a short preproANP sequence encoding the first 35 amino acids, with the 24 amino acid signal peptide (SP), linked in frame to the flag-EGFP cDNA. The signal peptide of preproANP is attached by a two amino acid spacer (-GS-) to the C-terminal of rat preproANP.

Microinjection and Electroporation

Similar to protocols of K. Ballmer-Hofer (Friedrich Miescher Institute, Basel, Switzerland), chimeric DNA (1 mg/mL; 3 μL) was added to 9.5 μL of autoclaved buffer and filtered through a Millipore 0.1-μm Ultrafree-MC centrifuge filter. An Eppendorf micropipette containing the DNA was connected to a 5171 micro-manipulator and 5246 transjector pressure control unit (Eppendorf) (holding pressure 80 hPa, injection pressure 60 hPa, and nuclear microinjection >300 ms). Up to 100 microinjections per dish were performed within 30 minutes at room temperature in HEPES buffer containing (in mmol/L) KCl 5, CaCl2 1, MgCl2 1, NaCl 118, glucose 2.5, HEPES 10; NaOH to pH 7.3, and sucrose to 290 mmol/kg H2O. Cells were usually examined 16 to 22 hours after microinjection. Success rates ranged from 0% to 3%. Electroporation (Gene Pulser II, Bio-Rad) yielded larger numbers of fluorescent myocytes per week. For each electroporation, 400 000 myocytes were suspended in PBS mixed with 4 to 8 μg of cDNA in a cuvette (4-mm electrode gap), and subjected to a 200-V shock (resistance 200 or 600 Ω, capacitance 800 μF). The cells were immediately plated in culture medium and returned to the incubator.

In Vivo Cell Labeling and Immunostaining

cDNA encoding the human transferrin receptor (V. Gerke, University of Munster, Munster, Germany) was co-microinjected in some cases, and cells were labeled 24 hours later by tetrastethyl-rhodamine-conjugated human transferrin (Molecular Probes) using a standard rhodamine filter cube (Nikon). Other labels (all from Molecular Probes) included Mitotracker Red, Syto-17, and Alexa 594 phalloidin. The cells were fixed with 2% paraformaldehyde and stored in ice-cold PBS. Cultures were pretreated with 1% normal serum, and the following primary antibodies were applied (4 days at 4°C): rabbit anti-rat ANP antisemur (ICH 9103, Peninsula Europe, Petersborough, UK) (dilution 1:1000), mouse anti-TGN38 monoclonal (ABR-MA3-063-R100, dilution 1:1000, Alexis Corp.), and mouse monoclonal anti-GFP gamma globulin (dilution 1:1000, Clontech). After repeated treatment with 1% normal serum, we detected the primary antibodies with Texas Red-X- or Alexa 594–labeled goat anti-rabbit or anti-mouse gamma globulin (Molecular Probes, 1:200 or 1:400). Separation of green and red fluorescence was achieved using Nikon filter cubes; green fluorescence, monochromatic excitation at 480±20 nm, dichroic mirror 495 nm,
Electron Microscopic Procedures

Cells on Cellocate coverslips (Eppendorf) were fixed in 1.6% glutaraldehyde followed by 2% OsO₄ postfixation. The specimens were then dehydrated in ethanol and embedded in Epon according to a standard procedure. For immunocytochemistry, cells on Cellocate-microgrid coverslips were fixed with 4% paraformaldehyde, washed, dehydrated in ascending concentrations of ethanol, and embedded in situ into LR White resin. After heat polymerization, the embedded cells were separated from the coverslips, and ultrathin sections were prepared (Leica Ultracut UCT). The sections were mounted on Formvar/carbon-coated nickel grids and processed, with minor changes, for postembedding immunogold labeling as previously described. Briefly, the grids with sections were pretreated with 1% normal goat serum, reacted for 17 hours with anti-GFP mouse monoclonal antibody (Clontech, diluted 1:300 or 1:500), and with a colloidal gold-conjugated goat anti-mouse IgG (Invitrogen, 15 nm) complex (particle size 12 nm). As controls, non–electroporated cells or cells electroporated without DNA were processed for immunolabeling in parallel, and cells from all samples were incubated in the absence of anti-GFP antibody. All preparations were stained with uranyl acetate and lead citrate, air-dried, and observed in a Philips CM 10 electron microscope at 80 kV using a 30- to 40-μm objective aperture.

Image Acquisition

A xenon lamp of a PTI DeltaRam monochromator illuminated the preparation at 480 or 575 nm (±6 nm bandwidth), using appropriate filter cubes (see Immunolabeling), a 100 oil fluorescence objective (NA 1.3), and exposure times of usually 500 ms (range 200 to 2000 ms). Two-color imaging was performed on live cells (EGFP and, e.g., Mito Tracker Red) or fixed cells (EGFP and, e.g., TGN-38). Black-and-white images (16-bit) were obtained with a quantum efficiency of 80% by a back-illuminated Princeton Instruments charge-coupled device (CCD) camera (TE/CCD-1000TKB, Visitron Systems). At ×100 magnification, each pixel corresponds to a 150×150-nm square. For repeated z-scans, 40 to 80 optical sections were obtained at 150- to 200-nm steps. For time-lapse studies, the preparation was repeatedly illuminated at intervals of 1 to 15 seconds for up to 60 minutes. The z-scan image stacks were dehazed using Huygens deconvolution software (Bitplane) by the iterative constrained Tikhonov-Miller procedure. The deconvoluted stacks were superposed in false colors: green for EGFP; red for TGN-38 or ANP (trans-Golgi network marker) (atrial myocyte, Figure 1d) and ANP (ventricular myocyte, Figure 1e). The presence of a signal peptide is efficiently directed into mobile vesicles (Figure 1c). The presence of a signal peptide is efficiently directed into mobile vesicles (Figure 1c). The presence of a signal peptide (Figure 1c) as well as of primary cultured atrial myocytes (Figure 1e). In atrial myocytes expressing proANP-10aa-EGFP, immunoelectron microscopy for EGFP specifically labels electron-dense vesicles of ~180 nm in diameter identified as secretory granules (Figure 2). No green fluorescence is detected in mitochondria or endosomes (not shown). Strong expression of green fluorescence also occurs in the perinuclear area that immunostains for TGN-38 (a trans-Golgi network marker) (atrial myocyte, Figure 1d) and ANP (ventricular myocyte, Figure 1e).
causes missorting of proANP-EGFP to the cytoplasm and the nucleus (not shown), presumably because of misfolding of proANP due to the narrow intermolecular distance between proANP and EGFP. The fusion protein proANP[1-11]-flag-EGFP, preceded by the signal peptide, is not sorted to secretory vesicles, but remains in the perinuclear area (atrial myocyte, Figure 1f). However, deletion of C-terminal or N-terminal proANP sequences, mutations of N-terminal Glu to Gln in positions 23 and 24, or permutation of proANP and EGFP (see Figure 1, top) does not preclude expression in mobile vesicles (Figures 3 and 4).

Cargo Signals Determine Vesicle Size, Shape, and Docking

The key observations are that mutations of N-terminal proANP change the size (Figures 3b, and 3c) and shape (Figures 3b, 3c, 4b, and 4c) of putative secretory vesicles and abolish their docking at the plasma membrane (Figures 3b, and 3c). Size histograms, established by tracking the mobile fluorescent vesicles containing proANP-10aa-EGFP, suggest the presence of smaller and larger vesicles, with apparent cross-sectional area (containing 60% of the fluorescence) of 2×2 and 3×3 pixels (Figure 3a). From calibrations with the green fluorescent microspheres, the vesicle diameters are deduced to be ≈120 and 175 nm, respectively. Smaller and larger mobile vesicles can be observed in the time-lapse studies (for example, see time-lapse 1 in the online data supplement available at http://www.circresaha.org). Docked vesicles also display smaller and larger sizes (for example, see time-lapse 2 in the online data supplement available at http://www.circresaha.org). The size histograms are preserved for vesicles containing the C-terminal–deleted proANP (Figure 3d) or proANP preceded by EGFP (Figure 3e) but are shifted to significantly larger sizes when the N-terminal of proANP is deleted (Figure 3b, χ²=32.92, df=3, P<0.001) or mutated (Figure 3c, χ²=10.09, df=3, P<0.02). The mobile vesicles containing N-terminal–deleted or –mutated proANP lose their spher-
The vesicular expression of a different fusion protein: a, proANP-10aa-EGFP; b, ΔproANP-10aa-EGFP; c, proANP(glu23,24=gln23,24)-10aa-EGFP; d, ΔproANP-10aa-EGFP; e, proANP[1-11]-F-EGFP-proANP[9-127]. Vesicle shapes, illustrated in Figure 4, are classified as round (circle), ovoid (ellipse), or floppy (star). Vesicles with fluorescence intensity as round (circle), ovoid (ellipse), or floppy (star). Vesicles with fluorescence intensity (purple, low intensity; red to white, high intensity).

Discussion

The following three points summarize this work. (1) Secretory vesicles can be imaged in live cardiomyocytes and analyzed for size, shape, velocity, and traffic path. (2) The genesis of spherical vesicles of well-defined size critically depends on the acidic calcium-binding N-terminal of proANP. (3) Mutation or deletion of this N-terminal creates larger floppy vesicles that do not dock at the plasma membrane.

Expression of GFP in Secretory Vesicles

This is the first imaging study of secretory vesicles in live cardiac myocytes. Western blots indicate that the fusion proteins remain intact, thus EGFP faithfully reports the presence of proANP or its mutated forms. The immunoelectron microscopy indicates that the EGFP, and thus the fused proANP, is localized in the secretory granules. The time resolution and sensitivity of the image acquisition are sufficient for tracking the vesicles up to once per second and for obtaining accurate estimates of their velocity. As shown with calibrated fluorescent microspheres, estimates of their size and shape can also be obtained.

Figure 3. Relationship between vesicle area, shape, and docking in 32 atrial myocytes. Note floppy vesicles and lack of docking for fusion proteins shown in panels b (N-terminal-deleted proANP) and c (N-terminal–mutated proANP). Histograms were established for area, shape, and velocity (columns 1, 2, and 4) of 245 mobile vesicles and for counts of all 286 mobile and 136 docked peripheral vesicles (column 3). Each row represents the vesicular expression of a different fusion protein: a, proANP-10aa-EGFP; b, ΔproANP-10aa-EGFP; c, proANP(glu23,24=gln23,24)-10aa-EGFP; d, ΔproANP-10aa-EGFP; e, proANP[1-11]-F-EGFP-proANP[9-127]. Vesicle shapes, illustrated in Figure 4, are classified as round (circle), ovoid (ellipse), or floppy (star). Vesicles with fluorescence intensity (>7 CCD units above background, median = 14 CCD units) were analyzed in detail (columns 1, 2, and 4). Docked versus mobile vesicles (column 3), regardless of intensity, were counted only in the cell periphery, in order not to include vesicles budding off the trans-Golgi network. The number of docked vesicles correlates with the number of mobile vesicles for vectors shown in panels a, d, and e (r = 0.71, P = 0.0006) but not panels b and c (r = 0.19, P = 0.29).

Figure 4. Examples of time-lapse images of vesicle shapes in atrial myocytes. The cDNA encoded as follows: a, SP-proANP-10aa-EGFP (normal vesicle, 500-ms exposure, interval 5 seconds); b, SP-ΔproANP-10aa-EGFP (large floppy vesicle, 800 ms, 10 seconds); b1, small floppy vesicle, 800 ms, 10 seconds; b2, small floppy vesicle, 800 ms, 10 seconds; b3, vesicle train in another myocyte, 800 ms, 3 seconds); c, SP-ΔproANP(glu23,24=gln23,24)-10aa-EGFP (floppy vesicle, 800 ms, 10 seconds); d, SP-ΔproANP-10aa-EGFP (normal vesicle, 500 ms, 2 seconds); e, SP-proANP[1-11]-F-EGFP-proANP[9-127] (normal vesicle, 1000 ms, 5 seconds). Calibration bar = 500 nm. Although these vesicles traveled over long distances, they are, to preclude movement artifacts, shown when hovering in one place. Vesicles are coded in false colors for fluorescence intensity (purple, low intensity; red to white, high intensity).
This study shows both smaller and larger fluorescent mobile vesicles, with estimated sizes (~120 and 175 nm in diameter) comparable to those of secretory granules in electron micrographs (eg, Reference 2). Smaller and larger vesicles with both high and low fluorescence intensity are observed, indicating that light intensity per se could not have been responsible for detecting two types of mobile vesicles. Both types are also present with two other expression vectors, one coding for a C-terminal–deleted proANP, the other for proANP[9-127] preceded by proANP[1-11]-flag-EGFP. The common feature is the presence of proANP[9-44], the acidic N-terminal amino acid sequence.

**Genesis of Floppy Vesicles Expressing N-Terminal–Mutated proANP**

Floppy vesicles were discovered in the myocytes expressing the N-terminal–mutated proANP. These vesicles are characterized by a strongly fluorescent core surrounded by a weakly fluorescent surrounding shape. One simple interpretation is that normally folded, endogenously produced proANP entraps and condenses the mutated fusion proteins in the central core, and that uncondensed mutated fusion protein is diluted in a fluid phase surrounding the central core. Hitchhiking of mutated prohormone with endogenously produced intact prohormone has previously been described for the granins.8 This concept is consistent with the finding that the histograms for vesicle sizes are shifted to larger diameters. There is a loss of a distinct size of the vesicles, possibly because of varying amounts of fluid trapped during their genesis in the trans-Golgi network.

**Figure 5.** Docking model for normal and floppy vesicles. a, condensed prohormone exhibits surface motifs known to be recognized by transmembrane macromolecules of the trans-Golgi network to allow for packaging of the secretory vesicle. Our results imply that these surface motifs also recruit docking receptors that will latch onto the docking proteins at the plasma membrane. b, Mutations in the acid calcium-binding domain inhibit condensation, increase the vesicle size by including a fluid phase, and thus cause floppiness of the vesicle. Uncondensed prohormone is unable to recruit docking receptors. Motor anchors are present as these floppy vesicles move at similar velocities. Condensed, endogenously produced, intact prohormone may still recruit docking receptors, but because these are too sparse or incorrectly situated, docking is abolished.

**Model Linking Shape and Docking of Atrial Secretory Vesicles to Cargo Signals**

The most striking feature of this work is the failure of floppy vesicles to dock at the plasma membrane. As a hypothesis, we propose a simple biophysical model that links the docking of secretory vesicles to the calcium-dependent condensation of its cargo (Figure 5). Normally folded prohormone aggregates in a dense matrix and is enwrapped in the trans-Golgi network by a tight-fitting membrane by recruiting transmembrane lipids and proteins (see Introduction). The results imply that the surface motifs of the condensed cargo also recruit docking receptors. These normal vesicles have little flexibility, offer a small contact area for vesicle motors, and are retained at the plasma membrane when encountering their docking proteins. Floppy vesicles arise when uncondensed mutated prohormone and its solvent surround the aggregate matrix. The floppy side of the vesicle may not recruit docking receptors, because surface motifs are absent. Thus, the probability of vesicle binding to the docking sites is strongly reduced. This model is compatible with and extends previous studies on sorting of secretory proteins aggregating in the trans-Golgi network.6–9,11,12,15 Ultimate proof demands the identification of as-yet-unknown docking receptors of the atrial granule and experimental evidence for their disappearance in floppy vesicles.

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**References**


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http://circres.ahajournals.org/content/89/3/e23

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2001/08/08/89.3.e23.DC1
Supplementary Material

Three time lapse studies are included (AVI format, PC, Windows 1998 or higher) and have been sent separately by e-mail to Matthew Bruns.

**Time lapse study 1:** Film F1.AVI (550KB)
Intense vesicle traffic in atrial myocyte expressing proANP-10aa-EGFP. Frame interval 5 sec, 500 msec exposure, film is sped up 150x. Calibration bar = 5 μm.

**Time lapse 2:** Film F2.AVI (531KB)
Docked vesicles in periphery of same cell. Frame interval 10 sec, 500 msec exposure, film is sped up 300x. Calibration bar = 5 μm.

**Time lapse 3:** Film F3.AVI (1247KB)
Lack of docking in atrial myocyte expressing N-terminal deleted proANP in vesicle trains. Frame interval 3 sec, 81 frames, 800 msec exposure. Film is sped up 90x. Calibration bar = 5 μm.