UltraRapid Communication

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

Alex J. Baertschi, Dominique Monnier, Uta Schmidt, Edwin S. Levitan, Stanislas Fakan, Angela Roatti

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 (glu23,24→glu23,24) 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 Electronic microscopy indicates that proatrial natriuretic peptide (proANP) is condensed in the trans-Golgi network2 and because proANP is cleaved only on release,4 secretory vesicles budding off the trans-Golgi network are already mature.5 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 plating 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, Centre Medical Universitaire, 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[1-11]-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 short preproANP fragment was amplified by polymerase chain reaction (PCR) from a pGEMI-preproANP vector (K.R. Chien, University of California at San Diego, San Diego, Calif). The plasmid for preproANP[1-11]-flag-EGFP was generated using two cloning vectors as templates, pEGFP-C1-preproANP and pcDNA3-signal peptide-proANP[1-11]-flag-EGFP. In a first step, we cloned a PCR fragment encoding a peptide from amino acids 9 to 127 of proANP into an XhoI site in the pEGFP-C1 vector (Clontech). We then amplified the signal peptide-proANP[1-11]-flag-EGFP fragment from pcDNA3-signal peptide-proANP[1-11]-flag-EGFP vector and cloned it into the pEGFP-C1-preproANP vector from which the EGFP cDNA had been previously excised. The preproANP-2aa-EGFP vector encodes a fusion protein where EGFP is attached to a two amino acid spacer (-GS-) to the C-terminal of rat preproANP. We performed two independent PCR reactions to amplify separately preproANP and EGFP full-length cDNAs. These fragments were fused together in an oriented manner, and the fused product was amplified by overlap extension PCR. The preproANP-10aa-EGFP vector encodes a preproANP-EGFP fusion protein truncated by a 10 amino acid spacer (-GPGINPPVPAT-) and was successfully expressed in secretory granules of PC-12 cells.

Western Blots

Twenty-four hours after transfection the HL-1 cultures were rinsed with HEPES buffer at 37°C, lysed, and sonicated. The extract was resolved on a 15% Acryl/Bisacrylamide-SDS gel for 2 hours at 100 V. The proteins were then transferred onto a nylon membrane (Millipore), and nonspecific sites were blocked. The membrane was incubated overnight at 4°C with polyclonal rabbit anti-EGFP antibody (Clontech, 1:4000), then for 1 hour at room temperature with HRP-conjugated anti-rabbit IgG (BioRad, 1:3000), and the EGFP bands were detected with the enhanced chemiluminescence (ECL) kit (Amersham).

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 Appendorf micropipette containing the DNA was connected to a 5171 micro-manipulator and 5246 transector 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 mosm/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 cells, and cells were labeled 24 hours later by tetramethylrhodamine–conjugated human transferrin (Molecular Probes) using a standard rhodamine filter cube (Nikon). Other labels (all from Molecular Probes) included Mito Tracker 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 antiserum (IHC 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-gamma globulin dilution (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,
emission filter 515±15 nm; red fluorescence, monochromatic excitation at 575±6 nm, dichroic mirror 595 nm, emission filter 630±30 nm. Controls included application of preimmune serum, no primary antibody, or preincubation of primary antibody with antigen.

**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-microgridded 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+IgM 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.

**Imaging Protocols**

Cells were electroporated on day 1 or microinjected on days 2 or 3 of culture. Between 16 to 50 hours after the DNA transfer, a green fluorescence–positive coverslip was placed in a specially designed chamber in HEPES buffer, and fluorescent myocytes were tagged by diamond-engraving the bottom of the slide. The chamber was then placed on a temperature-controlled (30°C to 35°C) stage of a wide-field Nikon DV200 Diaphot inverted microscope, and x-y coordinates for all tagged cells were determined for easy identification. After the image acquisitions, the cells were fixed and stored in PBS and darkness at 4°C. After immunolabeling and nuclear staining, the coverslip was placed again at a similar orientation on the setup for acquiring double z-scans of these cells.

**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, eg, Mito Tracker Red) or fixed cells (EGFP and, eg, 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 150x150-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 (MetaMorph, Universal Imaging Corp).

**Measurement of Vesicle Parameters**

Exactly the same analysis was applied to all fusion proteins. Vesicles in time-lapse images were zoomed twice 8 times (using edit-duplicate stack with zoom function, MetaMorph). For calibration purposes, green microspheres (Molecular Probes) with a diameter of 175 nm were imaged and analyzed the same way as the vesicles in live cells. As expected, the fluorescence intensity in the x-y plane is contained, after deconvolution, in a 2×2 pixel square (300×300 nm). Without deconvolution, the microspheres appear as a sphere of at least 750 nm in diameter. The emitted light thus diffuses over at least 25 pixels, with 58.6±0.3% (n=7 microspheres) of the total fluorescence appearing at optimal focus over a 3×3 pixel square. Accordingly, vesicle sizes in live myocytes were estimated—independently of shape—by counting, at optimal focus, the number of pixels in the x-y plane accounting for 60% of the total fluorescence intensity. For example, a small round vesicle may diffuse its light over >16 pixels and contain 60% of the fluorescence within a 2×2 pixel square. Vesicle shapes were classified as spherical, when 60% of the fluorescence intensity was spread over a square, as oval when 60% was spread over a rectangular area, and as irregular when 60% was spread over an obviously irregularly shaped area. Vesicle top velocity was measured by tracking the vesicle over its entire route. The fluorescence intensity of the vesicle was characterized by measuring the top pixel intensity (in CCD units) and normalized per 500 ms of exposure time. By calibration with precipitated GFP, it was estimated that 1 CCD unit per voxel corresponds to 150 fluorescent molecules. Constitutive vesicles containing uncondensed GFP would be >100 times less fluorescent, thus far below the threshold of detection. Docking of vesicles was attributed to peripheral vesicles remaining immobile at the cell surface over the entire recording period.

**Statistics**

Expression of 9 fusion proteins was studied in 137 atrial myocytes. Five types of fusion proteins were expressed in mobile vesicles (Figures 3 and 4). A total of 245 vesicles in 32 atrial myocytes with fluorescent intensities >7 CCD units were chosen for detailed analysis of size, shape, velocity, and intensity of the vesicles. A total of 422 vesicles (regardless of intensity) were counted as either docked or moving in the periphery of these 32 cells. Results were displayed as histograms. Statistically significant differences of distributions were analyzed by the χ² test.

**Results**

**Detection of Intact Fusion Proteins**

Figure 1 (top right) shows the Western blots for several of the fusion proteins that were expressed in the HL-1 cells, using an anti-EGFP antibody. Migration patterns in the gel indicate that the EGFP remains fused to the mutated proANP and that the vesicles revealed by EGFP imaging indeed contain the mutated proANP.

**Expression of EGFP in Mobile Vesicles Requires Signal Peptide and Part of proANP**

EGFP, or EGFP fused to the acidic flag peptide MDYKDDDK, is expressed in cytoplasm and nucleus of primary cultured rat atrial myocytes (Figures 1a and 1b). ProANP fused via a long linker, GPGLPPVAT, to EGFP and preceded by the signal peptide is efficiently directed into mobile vesicles of HL-1 cells (Figure 1c) as well as of primary cultured atrial (Figure 1d) and ventricular 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). The presence of a signal peptide is necessary but not sufficient for expression of proteins in mobile vesicles. Reducing the linker to two amino acids

Downloaded from http://circres.ahajournals.org/ by guest on April 19, 2017
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 3b1 and 3c1) and shape (Figures 3b2, 3c2, 4b, and 4c) of putative secretory vesicles and abolish their docking at the plasma membrane (Figures 3b, and 3c2) in atrial myocytes. 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 3a1). 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 3d1) or proANP preceded by EGFP (Figure 3e1) but are shifted to significantly larger sizes when the N-terminal of proANP is deleted (Figure 3b1, χ²=32.92, df=3, P<0.001) or mutated (Figure 3c1, χ²=10.09, df=3, P=0.02). The mobile vesicles containing N-terminal–deleted or –mutated proANP lose their spher-

Figure 1. Proproteins (top left) expressed in cardiomyocytes, Western blots (top right), and examples of live cells (bottom). EGFP indicates enhanced green fluorescent protein; F, flag; SP, signal peptide; proANP, proatrial natriuretic peptide; 10aa, 10 amino acid spacer; ΔN, N-terminal deleted; ΔC, C-terminal deleted; and 2aa, 2 amino acid spacer. Expected sizes of expressed proteins (in kilodaltons, without signal peptide) are 26.9 (EGFP), 41.8 (proANP-10aa-EGFP), 36.7 (ΔNproANP-10aa-EGFP), 29.3 (proANP[1-11]-F-EGFP), 42.1 (proANP[1-11]-F-EGFP-proANP[9-127]), and 40.8 (proANP-2aa-EGFP). Bottom panel: a, top, live atrial myocyte (EGFP); bottom, same cell immunostained for ANP (red) with residual EGFP (green). b, live atrial myocyte (F-EGFP) with red mitochondria and nucleoli. c, live HL-1–expressing proANP-10aa-EGFP. d, top, live atrial myocyte expressing proANP-10aa-EGFP; bottom, same cell double-stained for TGN-38 (red). e, left, live ventricular myocyte expressing proANP-10aa-EGFP; right, same cell immunostained for ANP (red). f, live atrial myocyte expressing proANP[1-11]-F-EGFP. Calibration bar=5 μm.

Figure 2. Electron microscopic localization of EGFP in secretory granules. a, live atrial myocyte expressing proANP-10aa-EGFP in mobile vesicles and in perinuclear area. b, same as panel a, showing colocalization of green fluorescence with monoclonal anti-EGFP (red). Note the higher sensitivity with immunostaining. Lack of immunostaining of some intensely green vesicles may be due to lack of penetration of antibody into condensed vesicle core. c, conventional electron microscopy of cultured atrial myocyte. d and e1, immunogold electron microscopy with the same monoclonal anti-EGFP antibody on atrial myocyte, expressing proANP-10aa-EGFP and previously identified in vivo. e2, cultured atrial myocyte stained as in panel e1, previously electroporated without DNA (control). Scale bar=5 μm in panels a and b; 250 nm in panels c, d, and e.
The following three points summarize this work. (1) Secre-
tory 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 immuno-
electron 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 suf-
cient 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.

Discussion
The following three points summarize this work. (1) Secre-
tory vesicles can be imaged in live cardiomyocytes and

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 docking (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-
D
CproANP-10aa-EGFP; b, Δ proANP-10aa-EGFP; c, proANP(gl23,24→gl23,24)-10aa-
EGFP; d, Δ proANP-10aa-EGFP; e, proANP[1-11]-F-EGFP-
proANP[9-127]. Vesicle shapes, illustrated in Figure 4, are classified
to round (circle), ovoid (ellipse), or floppy (star). Vesicles with fluo-
rescence intensity
as round (circle), ovoid (ellipse), or floppy (star). Vesicles with fluo-
rescence intensity
in Figures 3a, 3b, and 3d, yield similar results (not shown).

The top velocity reached by the vesicles (0.298±0.019
μm/s, n=245 vesicles) is affected neither by their shape nor
by their content (compare Figures 3b, 3c, 3d, and 3e, χ^2<1.15,
df=3, P>0.80). The slightly higher velocities
shown in Figure 3a are due to the presence of a larger
proportion of faster-moving peripheral vesicles versus
slow-moving perinuclear vesicles (results not shown).

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 sec-
onds); b, large floppy vesicle, 800 ms, 10 seconds); b2, small floppy vesicle, 800 ms, 10 sec-
ords; b3, vesicle train in another myocyte, 800 ms, 3 seconds); c, SP-proANP[gl23,24→gl23,24]-10aa-EGFP (floppy vesicle, 800
ms, 10 seconds); d, Δ proANP-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 bag of time-varying 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.

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.

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.

Acknowledgments
This study was supported by the Swiss National Science Foundation (to A.J.B. and S.F.), the de Reuter, Horten, Société Académique, Novartis, and Roche Research Foundations (to A.J.B.), and the National Institutes of Health (to E.S.L.). We thank J. Fakan for excellent technical assistance and Dr R. de Sousa for critical reading of the manuscript. Requests for materials (except flag-EGFP and preproANP-10aa-EGFP vectors) should be addressed to A.J. Baertschi.

References
Acid Prohormone Sequence Determines Size, Shape, and Docking of Secretory Vesicles in Atrial Myocytes
Alex J. Baertschi, Dominique Monnier, Uta Schmidt, Edwin S. Levitan, Stanislas Fakan and Angela Roatti

Circ Res. 2001;89:e23-e29
doi: 10.1161/01.01.00001095715

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
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.