Abstract—ANP (atrial natriuretic peptide) is widely recognized as an important vasorelaxant, diuretic, and cardioprotective hormone. Little is known, however, about how ANP-secretory vesicles form within the atrial myocytes. Secretory vesicles were visualized by fluorescence microscope imaging in live rat atrial myocytes expressing proANP–enhanced green fluorescent protein (EGFP), or N-terminal–mutated fusion proteins thought to suppress the calcium-dependent aggregation of proANP. Results showed the following: (1) aggregates of proANP and coexpressed proANP-EGFP recruited peptidylglycine α-amidating monoxygenase (PAM)-1, an abundant atrial integral vesicle membrane protein; (2) coexpressed N-terminal–mutated (Glu23,24→Gln23,24) and N-terminal–deleted proANP-EGFP inhibited recruitment of PAM-1 by up to 60%; (3) 4-phenyl-3-butenoic acid (PBA) (10 μmol/L), a pharmacological inhibitor of the luminal peptidylglycine α-hydroxylating monoxygenase domain of PAM proteins, inhibited recruitment of endogenous PAM-1 and of coexpressed pro-EGFP–PAM-1; (4) PBA had no effect on exocytosis of the potassium inward rectifier KIR2.1; (5) PBA induced a deformation of the secretory vesicles but did not inhibit docking. These findings suggest that recruitment of PAM-1 to secretory vesicles depends on intact N-terminal proANP and on the luminal domain of PAM-1. Conversely, PAM-1 participates in shaping the proANP-secretory vesicles. The full text of this article is available online at http://circres.ahajournals.org.

Key Words: atrium ■ EGFP ■ in vivo imaging ■ PAM ■ proANP ■ secretory vesicles

A trial natriuretic peptide (ANP), originally discovered by de Bold,1 is an endogenous hypotensive hormone involved in the regulation of blood pressure and fluid homeostasis.2,3 ANP is secreted from the cardiac atria in response to cardiac stress such as oxygen deficiency and mechanical overload (see Jiao and Baertschi4 for citations). Appropriately, ANP relieves the heart from hypoxic vasoconstriction of the pulmonary circulation and also helps protect the heart from mechanical overload.2–4 ANP and brain natriuretic peptide have become important clinical markers of cardiac hypertrophy and failure. Accordingly, there are thousands of studies on ANP release and action, in both animal and man.

In contrast to this wealth of knowledge, the cell biology of ANP-containing secretory vesicles is far less understood. ANP is synthesized as an inactive precursor, preproANP. A 24-aa signal sequence directs the nascent amino acid chain into the lumen of the endoplasmic reticulum, wherefrom it is processed through the Golgi stacks to the trans-Golgi network (TGN). The prohormone proANP is not cleaved but remains intact during its journey from the TGN to the plasma membrane.5 Sorting of proANP to secretory vesicles may be accomplished by calcium-dependent aggregation of proANP5,6 within electron-dense granules in the TGN.7 By analogy with other secretory systems,8–12 integral membrane proteins of the TGN may recognize the surface layer of the proANP core and enwrap it with TGN membrane to form the secretory vesicle. Our previous study indicates that mutations of the N-terminal calcium-binding domain of proANP results in deformed vesicles that no longer dock at the plasma membrane.13 This raises the question about the identity of the proteins that are recruited to shape and dock the secretory vesicles.

Although proteins involved in secretory vesicle budding and fusion are now well characterized in yeast14 those of the cardiac ANP-secretory pathway are still largely unknown. Those known to play important roles in other mammalian secretory systems, such as vesicle associated protein and SCAMP secretory carrier–associated protein,14,15 could not be detected, by our preliminary studies, in significant amounts on atrial proANP vesicles. We found that peptidylglycine α-amidating monoxygenase (PAM), an abundant protein family in the cardiac atrium,16–19 is localized on atrial proANP–enhanced green fluorescent protein (EGFP)–expressing vesicles and could thus be a viable candidate. PAM
posttranslationally activates approximately half of all mammalian neuropeptides by converting their COOH-terminal glycine into an essential amide moiety (see Prigge et al.16 for review). PAM catalyzes this reaction in two consecutive steps via peptidylglycine α-hydroxylating monoxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase. Several forms of PAM mRNA result from alternative splicing of the single copy rat PAM gene. PAM-1, the longest form, is composed of the PHM catalytic domain, a noncatalytic domain (exon A), the peptidyl-α-hydroxyglycine α-amidating lyase catalytic domain (all within the vesicle lumen), a transmembrane domain (TM), and a C-terminal domain (CD) that extends into the cytoplasm. Deletion of exon A yields PAM-2, and further deletion of TM gives rise to soluble PAM-3 within the vesicle lumen (Figure 1C). In the heart atrium, most of the PAM-1 does not seem to be cleaved into smaller protein products.17–20 So far, there is no known substrate for PAM in atrial vesicles; thus other functions of PAM have been explored. Because the lumenal catalytic domain of PAM is pH sensitive, PAM might play a role in signaling lumenal conditions to the cytosolic proteins.21 Mutation of the PAM-CD at juxtamembrane sites eliminates the ability of PAM to bind to the PAM C-terminal interacting protein and to modify regulated secretion.22–24 The pH-dependent aggregation of PAM25 could play a role in its segregation to secretory granules. If PAM interacted with lumenal proteins of the TGN, such as proANP, PAM could be involved in forming the atrial secretory vesicles.

The purpose of this study was to determine whether PAM proteins were implicated in shaping and docking the atrial proANP vesicles. We answered the following questions. Are PAM proteins recruited to proANP-EGFP–expressing secretory vesicles? Do mutations of the N-terminal calcium-binding sequence of proANP suppress recruitment of PAM? Does a pharmacological inhibitor of PAM inhibit recruitment of PAM, deform the secretory vesicles, or suppress docking? The imaging of single atrial vesicles13 in live atrial myocytes was crucial for answering some of these questions.

### Materials and Methods

#### Primary Culture of Atrial Myocytes
Atrial appendage cells from neonatal rats (2- to 4-days old; Sprague-Dawley) were cultured as described previously13,26 on 22-mm glass slides in F10 medium. Late pregnant rats were obtained from Charles River Laboratories (L’Arbresle, France) or from the University of Geneva. Experiments were conducted according to the Guide for Care and Use of Laboratory Animals (with approval of the University Ethics Committee and the State of Geneva Veterinary Office).

#### Plasmid Constructs
Three pcDNA3-EGFP expression vectors were used for encoding preproANP-10aa-EGFP27 (a gift of Edwin Levitan, University of Pittsburgh, Pa), preproANP (Gln23,24→Glu23,24)-10aa-EGFP, and preproANP(45–127)-10aa-EGFP13 (Figure 1A). The 10-aa sequence forms the spacer. Electroporation of these plasmids was successfully performed with a Gene Pulser II (Bio-Rad). From calibrations with EGFP protein, and the number of prohormone molecules contained in a dense core secretory vesicle,28 the mutated fusion proteins are estimated to be coexpressed in a ratio of at least 1:2 with endogenous proANP.

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**Figure 1.** Constructs and antisera used in this study. A. Wild-type proANP-EGFP and mutant fusion proteins expressed in atrial myocytes. SP indicates signal peptide for proANP; 10aa, 10-aa spacer. Numbering refers to wild-type proANP; N, deletion of calcium binding N-terminal of proANP13; 23,24(Glu→Gln), mutations in N-terminal proANP13 of glutamic acids involved in calcium binding. B, pro-EGFP–PAM-1 fusion protein encoded by a new construct described in Materials and Methods. Numbering starts with first amino acid of prosequence. SP indicates signal peptide for PAM-1; pro, 10-aa (FRSPLSVFKR) pro-sequence of PAM-1, which is followed by 4 aa (FGPG) including the first Apa site of the cDNA (first arrow); EGFP is followed by a 10-aa spacer (GGPSIN-PPVA) including the second Apa site (second arrow). A indicates exon A domain. From Prigge et al.,16 there is no potential cleavage site between EGFP and PHM. C, main proteins of the PAM family in atrium. Antisera (as) used in this study included as475 (anti-PHM); as629 (anti–PAM-1; anti–exon A); and as571 (anti–C-terminal domain, CD-as).33

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A new construct, encoding EGFP-PAM-1 preceded by the PAM-1 prosequence (Figure 1B), was made in several steps from the pCIneo plasmid (Promega Corporation, Madison, Wis) containing PAM-1 cDNA (a gift of R. Mains, University of Connecticut, Farmington). First, an Apal restriction site was inserted by PCR between the prosequence and the PHM domain of full-length PAM-1 cDNA. Two primer pairs: prosequence and the PHM domain of full-length PAM-1 cDNA. Two primer pairs: prosequence antisense (aatatcagaacctcttaaagacaga); and PHM sense (atatat) primer pairs: prosequence and the PHM domain of full-length PAM-1 cDNA. Two primer pairs: prosequence antisense (aatatcagaacctcttaaagacaga); and PHM sense (atatat)

To overlap the PHM domain, the mutagenesis primer Apal 10-aa sense (tatagggcccagatccagtcttcaat), prosequence antisense (attagggcccaaccttgaactccagtagcatttaaagaaact) was designed with the signal peptide and the prosequence, and insert 21 new nucleotides overlapping the 5′-PHM domain. These two fragments were ligated using the engineered Apal restriction site (shown in italics). The resulting fragment was subcloned in the pCneoPAM-1 plasmid, replacing the BglII/EcoRV cassette (pCneoPAM-1/Apal). Second, to create a 10-aa spacer upstream of the PHM domain, the mutagenesis primer Apal 10-aa sense (tatagggcccagatccagtcttcaat), prosequence antisense (attagggcccaaccttgaactccagtagcatttaaagaaact) was designed to overlap the Apal restriction site on the pCneoPAM-1/Apal plasmid and insert 21 new nucleotides overlapping the 5′-PHM domain. Together with the EcoRV primer, a new Apal-10aa/EcoRV fragment was amplified by mutagenesis PCR. This fragment was subcloned in the pCneoPAM-1/Apal plasmid to generate the pCneoPAM-1/Apal10aa plasmid. Third, EGFP cDNA was amplified from the pEGFP-N1 plasmid sequence (Clontech, Palo Alto, Calif) by PCR with a primer pair, allowing to create Apal restriction sites at the 5′ and 3′ ends of the cDNA and to mutate the ATG and TAA codons. This fragment was subcloned in the pCneoPAM-1/Apal and pCneoPAM-1/Apal10aa plasmids. The two plasmids, pCneoPAM-1/Apal EGFP and pCneoPAM-1/Apal10aa EGFP, were electroporated in atrial myocytes and HL-1 cells. Only the construct containing the 10-aa spacer gave rise to EGFP expression; the green fluorescence being localized to ANP-secretory vesicles (Figure 2C).

**Treatment of Atrial Myocytes With 4-Phenyl-3-Butenoic Acid**

Vehicle or 4-phenyl-3-butenoic acid (PBA) (10 μmol/L), the most potent irreversible, mechanism-based PHM inactivator known,29 was added to new culture medium each day. As a control experiment, the effects of PBA were tested on another regulated secretory system, the inwardly rectifying potassium channel Kir2.1,30,31 unrelated to pro-ANP and without interactions with PAM-1.32 The exocytosis of EGFP-Kir2.1 was compared in 27 atrial myocytes treated with PBA or vehicle, as described in the legend to Figure 6.

**Imaging**

**Protocols**

Between 16 and 50 hours after electroporation, the cultures were placed in HEPES-containing buffer (mmol/L: KCl, 5; CaCl2, 1; MgCl2, 1; NaCl, 118; glucose, 2.5; HEPES, 10; NaOH to pH 7.3; sucrose to

![Figure 2. Expression of EGFP in secretory vesicles of neonate rat atrial myocytes electroporated with plasmids described in Figure 1A and 1B. A, Serial horizontal optical sections cut through secretory vesicles (A1, A2, and A3) in an atrial myocyte expressing wild-type proANP-EGFP and immunostained for PAM-1 (one optical section of myocyte shown in A4). Note that most vesicles and TGN are double-stained (yellow; example A3), whereas some vesicles show only green fluorescence (A1) or only PAM-1 (red, A2). B, Central portion of atrial myocyte expressing wild-type proANP-EGFP and immunostained for ANP (wide-field image). Nucleus is stained in blue (DAPI), and surrounding TGN and most vesicles are stained yellow-orange (fusion of EGFP, green; and ANP-immunostaining, red). C, Optical section through an atrial myocyte expressing pro-EGFP–PAM-1 and immunostained for ANP (one optical section of myocyte shown in A4). Note that most vesicles and TGN are double-stained (yellow; example A3), whereas some vesicles show only green fluorescence (A1) or only PAM-1 (red, A2). B, Central portion of atrial myocyte expressing wild-type proANP-EGFP and immunostained for ANP (wide-field image). Nucleus is stained in blue (DAPI), and surrounding TGN and most vesicles are stained yellow-orange (fusion of EGFP, green; and ANP-immunostaining, red). D, Staining in Western blots for neonate atrial myocyte cultures (A1 to A6) by anti–PAM-1 as629 (D1), anti–CD-PAM antiserum as571 (D2), and anti-PHM antiserum as475 (D3). Note band at an apparent molecular mass of 125 kDa corresponding to PAM-1 in D1 and two bands (~125 to 130 kDa and 110 kDa) corresponding to PAM-1 and PAM-2 in D2 and D3. A third very faint band (just ~90 kDa) represents PAM-3 in D3. Note lack of specific staining for ventricular cultures (V1 and V2). Cultures A4 and A6 were treated for 72 hours by 10 μmol/L PBA (for rationale, see Figure 4C and corresponding text). Control electroporation with EGFP-N1 plasmid yields green fluorescence in cytoplasm and nucleus as expected (not shown). The optical sections were obtained after Huygens deconvolution of wide-field images or directly acquired using a spinning wheel confocal head (see Materials and Methods).
290 milliosmol/kg H2O on a temperature-controlled (30°C to 35°C) stage of a wide-field Nikon-DV200 Diaphot microscope. After imaging, the cells were fixed for 15 minutes in 2% paraformaldehyde and stored in PBS and darkness at 4°C until immunolabeling.

**Acquisition**

Image acquisition was performed with Metamorph software (Universal Imaging) at a monochromatic excitation of 480 nm (EGFP) or 575 nm (Texas Red) (±6-nm bandwidth), with illumination times of usually 500 ms (range 300 to 1000 ms). Forty to 80 optical sections were obtained at 150-nm vertical steps. The z-scan image stacks were deconvoluted using Huygens software (Bitplane) by the iterative constrained Tikhonov–Miller procedure and superposed by Metamorph software. Confocal optical sections were also acquired directly using a laser excitation of 488 nm and 568 nm with a Nikon Diaphot 300 inverted microscope with a NA 1.3 oil objective, a QLC100 spinning wheel confocal head (VisiTech International), and a CoolSnap HQ cooled charge-coupled device (CCD) camera (Visitron Systems). The pixel size (at 2×2 binning) in the x-y plane was 129 nm.

**Measurement of Shape and Docking**

The shape of live, peripherally located vesicles in time lapses or z scans was analyzed by zooming first at 150% and then at 800% (using edit-duplicate-stack-with-zoom function; MetaMorph). For each vesicle, gamma and contrast were adjusted from 1 to 5.43 and from 50 to 70, respectively, before automatic contour fitting. Each vesicle was coded by a number and classified twice by two observers as round when more than 50% of all contours were round. In controls, between 50% and 60% of vesicles were classified as round. Vesicles were classified as docked when the vesicles remained immobile in the periphery of the cell and over the total recording period; otherwise, they were classified as moving. A peripheral location was chosen so that secretory vesicles would not be confused with vesicles bulging from the TGN; the vesicles were close to the cell membrane, thus increasing the likelihood that they were docked. It was unavoidable that some vesicles that were close to the membrane but not docked could not be distinguished from truly docked vesicles.

**Immunostaining**

To study colocalization of green fluorescence with ANP, PAM-1, or CD-PAM, the fixed cultures were pretreated with 1% normal serum and one of the following primary antibodies was applied (4 days at 4°C): rabbit anti-rat ANP antiserum (IHC9103; Peninsula Europe) and one of the following primary antibodies was applied (4 days at 4°C): rabbit anti-rat ANP antiserum (IHC9103; Peninsula Europe) and one of the following primary antibodies was applied (4 days at 4°C): rabbit anti-rat ANP antiserum (IHC9103; Peninsula Europe) and one of the following primary antibodies was applied (4 days at 4°C): rabbit anti-rat ANP antiserum (IHC9103; Peninsula Europe). For example, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (D-1306; Molecular Probes). For example, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (D-1306; Molecular Probes). For example, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (D-1306; Molecular Probes). For example, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (D-1306; Molecular Probes).

**Statistics**

The analysis of colocalization and of vesicle shape was performed on a total of 2992 vesicles in 335 atrial myocytes. The analysis of vesicle docking was performed on a total of 4813 vesicles in an overlapping population of 170 myocytes. The analysis was divided into two parts: (1) colocalization of proANP-EGFP or pro-EGFP–PAM-1 fusion proteins with PAM or ANP immunostaining; and (2) tests with the PHM inhibitor PBA. Results were displayed as histograms of weighted averages. Differences were analyzed for statistically significant differences by ANOVA tests on ranked data using SAS software from the SAS Institute (Carey, NC), the nonparametric Mann–Whitney U test, or χ² test.

**Results**

**Characterization of Experimental System**

We used a cDNA construct encoding a fusion protein of proANP-EGFP that was localized by immunoelectron microscopy in dense core vesicles of atrial myocytes. A potential protease site at the C terminus of the proANP was replaced with a 10-aa spacer (GPGLNPPVPAT) to prevent cleavage of proANP-EGFP. As control, we electroporated myocytes with the proANP-EGFP expression vector and labeled the cells with an antibody directed against ANP. The great majority of cells, 88.8±2.3%, were labeled by EGFP as well as by anti-ANP antibody. An example is shown in Figure 2B, where the superposition of EGFP with ANP (red) yields yellow-orange vesicles and TGN surrounding the DAPI-stained (blue) nucleus. To further address the question of cleavage, the proANP-EGFP was expressed in HL-1 atrial myocytes. Western blots for EGFP showed a band corresponding to the intact proANP-EGFP fusion protein, confirming our previous study (not shown). Analogous results were obtained with the pro-EGFP–PAM-1 expression vector (eg., Figure 2C; Western blot not shown). Furthermore, Western blots for endogenous PAM-1 for atrial but not ventricular culture extracts showed a major band ~125 kDa (Figure 2D1). Blots for CD-PAM displayed two major bands for atrial but not ventricular culture extracts ~125 kDa and 110 kDa (Figure 2D2). A nonspecific band of lower molecular mass appeared for all extracts. Blots for PHM showed, in four atrial extracts, a weak band ~125 to 130 kDa, a strong band ~110 kDa, and a very faint band ~90 kDa (Figure 2D3). Previous reports indicate that these bands correspond to PAM-1 (Figure 2D1), PAM-1 and PAM-2 (Figure 2D2), and PAM-1, PAM-2, and PAM-3 (Figure 2D3). Small amounts of PAM-3 in atrium (Figure 2D3) have been reported previously. Thus neither proANP-EGFP, nor pro-EGFP–PAM-1, nor endogenous PAM proteins are cleaved to a major degree in our culture system, as anticipated from previous reports.

**Effects of ProANP Mutations on Colocalization With PAM Proteins**

We tested the hypothesis that point mutations or total deletion of the calcium-binding sequence of proANP would disrupt the aggregation of proANP and thus inhibit recruitment of PAM proteins. In 47 myocytes (435 vesicles), we examined whether these mutations suppressed the colocalization with PAM-1 (Figure 3A) and in 46 myocytes (469 vesicles), the colocalization with CD-PAM (Figure 3B). A wild-type proANP-EGFP–expressing myocyte is shown in Figure 2A, indicating a high degree of colocalization with PAM-1. Overall, there was a majority of vesicles (83.9%) where proANP-EGFP was colocalized with PAM-1 and a minority
where proANP-EGFP was expressed alone (Figure 3A1). Point mutations (Glu23,24→Gln23,24) or deletion of the calcium-binding N-terminal 44 aa reduced the proportion of PAM-1 colabeled vesicles to 58.7% (mean±SEM, n=3) and 33.2% (mean±SEM, n=38.1; ANOVA). Percentage of colabeled vesicles decreases significantly in A2 and A3 relative to wild type (\(P<0.01\), \(P<0.001\), \(\chi^2\) test; \(P<0.015\) for N-terminal deletion, ANOVA). B, labeling by anti–CD-PAM as571 of 469 secretory vesicles containing wild-type, mutated, and N-terminal–deleted proANP-EGFP (see above). Percentage of colabeled vesicles decreases slightly from B1 to B3 (\(P<0.05\), \(\chi^2\) test).

(16.1%) where proANP-EGFP was expressed alone (Figure 3A1). Point mutations (Glu23,24→Gln23,24) or deletion of the calcium-binding N-terminal 44 aa reduced the proportion of PAM-1 colabeled vesicles to 58.7% (\(\chi^2=10.3; DF=1; P<0.01\)) and 33.2% (\(\chi^2=38.1; DF=1; P<0.001\)), respectively (Figure 3A2 and 3A3). Examples for such vesicles are shown in the top panels of Figure 3A. A slight decrease in anti–CD-PAM labeling was observed with the proANP mutations; a majority of vesicles being double-labeled: 92.2% for intact proANP; 89% for Glu23,24-mutated proANP (Figure 3B2); and 84.7% for N-terminal–deleted proANP (Figure 3B3; \(\chi^2=4.3; DF=1; P<0.05\)). Because the CD-PAM antiserum labels PAM-1 and PAM-2, an estimate was made whether this decrease in colocalization could also have been attributable to PAM-2. In Western blots, PAM-1 accounts for 27.5±9.6% (mean±SEM, n=3) of PAM-1 plus PAM-2. If recruitment of PAM-1 alone was affected by deletion of N-terminal proANP, it is calculated that CD-PAM should colabel 76.9% of the green fluorescent vesicles, close to the 84.7% observed. The difference is within the margin of error of the measurements.

**Effect of PHM Inhibitor PBA on Recruitment of PAM-1**

We next tested whether PBA\(^{29,34}\) would modify recruitment of PAM-1 to secretory vesicles, in a total of 26 atrial myocytes (834 vesicles) expressing proANP-EGFP and treated for 72 hours with 10 \(\mu\)mol/L PBA or vehicle. Figure
4A1 shows that PBA strongly increased the mean percentage of PAM-1-negative green fluorescent vesicles per cell (\(\chi^2=12.3; DF=1; P<0.01\)). Figure 4A2 illustrates for individual myocytes that PBA produced a major shift toward PAM-1-negative vesicles, indicating a loss of recruitment of PAM-1. Microphotographs of Figure 4A3 illustrate that in a vehicle-treated myocytes (control), all EGFP-positive vesicles were labeled by anti–PAM-1 antiserum. In the PBA-treated myocyte, a few EGFP-positive vesicles were also labeled by anti–PAM-1 antiserum, but even strongly EGFP-positive vesicles were not stained at all. As a control, Figure 4B shows that PBA did not decrease the maximal immunofluorescence intensity for PAM-1 in TGN and vesicles; thus the difference in PAM-1 immunostaining of the vesicles in Figure 4A was not attributable to major changes in staining procedures. In another control, Western blots show that PBA had little effect on CD-PAM staining of secretory vesicles. Following PBA treatment, 83.2±7.3% of proANP-EGFP–expressing vesicles per cell were colabeled by CD-PAM antiserum (\(n=7\) myocytes, 276 vesicles), similar to the percentage shown for vesicles expressing N-terminal truncated proANP-EGFP (Figure 3B3).

**Effect of PBA on Recruitment of Pro-EGFP–PAM-1**

In another experimental approach, we tested whether recruitment of pro-EGFP–PAM-1 to proANP vesicles was also affected by PBA. Pro-EGFP–PAM-1 was expressed in atrial myocytes, and these were treated for 3 days with PBA or vehicle (Figure 5). Most, if not all, green fluorescent vesicles were double-labeled for ANP, in both the controls and PBA-treated myocytes, presumably because of the extremely strong signal for ANP immunostaining. An example for a control is shown in Figure 5C; and for a PBA treated myocyte, in Figure 5A. However, PBA significantly changed the distribution of 180 secretory vesicles analyzed in 18 atrial...
myocytes (Figure 5B1), where the fluorescence intensity of EGFP was plotted as a function of ANP immunostaining. PBA thus reduced by approximately half the green fluorescence intensity relative to ANP (Figure 5B2).

Control Experiment Testing the Effect of PBA on Another Secretory System: Exocytosis of KIR2.1
Would PBA indiscriminately affect other secretory systems? As is the case for many transporters and channels, the exocytosis of the potassium inward rectifiers KIR2.x can be regulated (see Zitron et al30 and Malinowska et al31 for citations). The choice of KIR2.1 has the advantage that none of its numerous interacting partners includes PAM, as recently shown.32 Constructs encoding fusion proteins of KIR2.1 with EGFP were electroporated into atrial myocytes (Figure 6). After 3 days, green fluorescence was equally detectable in the plasma membrane of vehicle- or PBA-treated myocytes (examples in Figure 6A1 and 6A2). Quantification with line scans (Figure 6B1 and 6B2) showed there was no significant difference in membrane relative to intracellular green fluorescence intensity between 13 controls and 14 PBA-treated myocytes (Figure 6C1 and 6C2).

Effect of PBA on Shape and Docking of ProANP Vesicles in Live Myocytes
We tested in 136 myocytes, on 798 proANP-EGFP vesicles, whether 10 μmol/L PBA would change vesicle shape or docking. The proportion of deformed vesicles in vehicle-treated cells remained stable: 42.2% at 48 hours; 51.4% at 72 hours; and 52.2% at 96 hours (Figure 7A; examples in top panels). After 3 days of treatment, PBA produced a major change in vesicle shape, in a majority of spherical vesicles, to irregular (deformed) vesicles. The proportion of deformed vesicles (Figure 5B1), where the fluorescence intensity of EGFP was plotted as a function of ANP immunostaining, PBA thus reduced by approximately half the green fluorescence intensity relative to ANP (Figure 5B2).

Figure 5. Inhibitor (PBA) of PHM decreases recruitment of pro-EGFP–PAM-1 to ANP-containing vesicles. Atrial myocytes expressing pro-EGFP–PAM-1 were treated for 72 hours with 10 μmol/L PBA or vehicle (control), fixed, and immunostained for ANP; stacks of confocal optical sections were obtained for green and red fluorescence and superposed to check for double-labeling of the vesicles. For each of 18 myocytes (9 controls, 9 PBA-treated), 10 vesicles that displayed the highest green fluorescence intensity were analyzed for coexpressed red fluorescence intensity. The background-corrected fluorescence intensities (in CCD units) were normalized to the exposure time used in the image acquisition. The intensity of laser excitation was the same throughout. A, Confocal optical section through a PBA-treated myocyte acquired at 488-nm excitation (top, pro-EGFP–PAM-1) and at 568 nm (bottom, ANP). Note that practically all vesicles are double-labeled, even though the cell was treated with PBA. Because this was true for all PBA-treated myocytes and the controls, green and red fluorescence intensities were quantified in B. B1, Plot of green fluorescence intensity of pro-EGFP–PAM-1 as a function of red fluorescence intensity (ANP) for each of 180 vesicles. Note significantly different distribution for controls and PBA treatment ($P<0.01; \chi^2=12.1; DF=3$). Distributions remained significantly different ($P<0.001; \chi^2=29.3; DF=3$) when the ANP values ranged from 250 to 780 CCD units/sec. Thus, for a similar ANP content, the vesicles recruited significantly less pro-EGFP–PAM-1. B2, Mean pro-EGFP–PAM-1/ANP ratio for 90 control vesicles and 90 vesicles from PBA-treated myocytes. Note significant decrease with PBA ($*P<0.05$, Mann–Whitney $U$ test).
vesicles increased from 52.1% at 48 hours to 82.4% at 72 hours ($\chi^2=25.5; DF=1; P<0.001$) and 73.7% at 96 hours ($\chi^2=9.1; DF=1; P<0.01$). We then tracked the movements of 4813 secretory vesicles in a total of 170 live myocytes (Figure 7B). There was no difference in the proportion of mobile vesicles of controls versus PBA-treated cells during the experiment: 79.9% versus 82.5% at 48 hours; 81.9% versus 84.8% at 72 hours; 85.2% versus 83.5% at 96 hours.

**Discussion**

ProANP, PAM-1, and PAM-2 are highly abundant proteins of the cardiac atrium, contained in the lumen and membrane, respectively, of the same secretory vesicles. The reasons for this intriguing, intimate relationship between proANP and PAM have remained unclear. The aims of this work were to test in living myocytes whether the calcium-dependent aggregation of proANP\(^5,6\) and the lumenal domain of PAM were required to recruit PAM-1 and PAM-2 to the vesicle membrane and, conversely, whether PAM proteins contributed to the genesis and docking of proANP vesicles. We show that recruitment of PAM-1, but not PAM-2, depends on an intact calcium-binding N-terminal sequence of proANP. We go on to show that PBA, a pharmacological inhibitor of PAM, inhibits recruitment of PAM-1 but not PAM-2, induces a deformation of the secretory vesicles, and has no effect on docking. These novel findings, summarized in Figure 8, were obtained on neonate atrial myocytes in culture, a favorable model for studying the cardiac secretory pathway. \(^13,17–19,36\)
Recruitment of PAM-1 to Secretory Vesicles Is Inhibited by Mutations of the Calcium-Binding Domain of ProANP and by PBA

How do PAM-1 and PAM-2 target the atrial secretory vesicle membrane? At least two possibilities exist, or may coexist. First, the protein kinase PAM C-terminal interacting protein can bind tightly to the C-terminal cytosolic juxtamembrane domain of PAM-1 and PAM-2, and phosphorylate PAM-1 on Ser949. This may facilitate the aggregation of PAM-1 (see below) and recruitment of PAM-1 to secretory vesicles in AtT-20 cells. Ser949 phosphorylation also favors retrieval of PAM-1 from the plasma membrane onto endocytotic vesicles and/or retention of PAM-1 in the TGN. Second, it is reported that PAM-3, the soluble form of PAM-1 and PAM-2, precipitates either by itself or with luminal cargo proteins at ionic conditions prevailing in the TGN, suggesting that the luminal domain of PAM-1 could do likewise. From our data and these previous studies, we propose that PAM-1 is recruited to the proANP aggregate, either by coprecipitating with proANP on the surface of the aggregate or by recognizing surface motifs of the aggregate (Figure 8A). We base this suggestion on the following findings. When aggregation of proANP is perturbed by deletion or mutation of its calcium-binding domain, recruitment of PAM-1 to secretory vesicles is strongly diminished (Figure 3A), as illustrated in Figure 8B. The recruitment of PAM-1 is also diminished by PBA, as
shown by two different experimental approaches. First, PBA reduces the colocalization of proANP-EGFP and PAM-1 on secretory vesicles (Figure 4A); and second, PBA reduces the colocalization of pro-EGFP-PAM-1 and ANP on secretory vesicles (Figure 5B). Interestingly, PBA has no effect on the exocytosis of KIR2.1 (Figure 6), suggesting that the actions of PBA may be confined to PAM. The mechanism of action of PBA in our experiments is not yet known. It is not established whether PAM-1 is functionally active within the TGN or the secretory vesicles, the more so because a substrate has not yet been identified. Changes in enzymatic activity of PHM in extracts were not measured here, because we would not have been able to distinguish between any changes occurring in TGN or secretory vesicles; the purification of secretory vesicles from atrial cell cultures has remained elusive so far. As an alternative possibility, the action of PBA on PHM may alter the conformation of PAM-1 (illustrated in Figure 8C) and thus inhibit the interaction between PHM and N-terminal proANP. The mechanism of action of PBA in our experiments is not yet known. It is not established whether PAM-1 is functionally active within the TGN or the secretory vesicles, the more so because a substrate has not yet been identified. Changes in enzymatic activity of PHM in extracts were not measured here, because we would not have been able to distinguish between any changes occurring in TGN or secretory vesicles; the purification of secretory vesicles from atrial cell cultures has remained elusive so far. As an alternative possibility, the action of PBA on PHM may alter the conformation of PAM-1 (illustrated in Figure 8C) and thus inhibit the interaction between PHM and N-terminal proANP.

The recruitment of PAM-2 apparently does not depend on the aggregation of proANP. This is based on the finding that the staining of secretory vesicles with CD-PAM antiserum decreases only slightly with deletion or mutation of the N-terminal sequence of proANP (Figure 3B). The CD-PAM antiserum recognizes both PAM-1 and PAM-2. This report shows that PAM-1 accounts for 27.5% of the total amount of PAM-1 and PAM-2 in atrial myocytes, confirming previous studies.19,35,36 The slight decrease in staining can thus be attributed entirely to the loss of recruitment of PAM-1. The cytosolic routing motifs of PAM-2 (citations in24) may be sufficient for targeting PAM-2 to secretory vesicles, irrespective of potential interactions with proANP. The difference between PAM-1 and PAM-2 is the exon A sequence in PAM-1, located downstream of the PHM domain. It is speculated that the presence of exon A facilitates the interaction between PAM-1 and proANP. A potential influence of exon A on the conformation of PHM would explain why PBA inhibits recruitment of PAM-1 but not PAM-2 to secretory vesicles, even though PBA has the same site of action (PHM) in PAM-1 and PAM-2. Alternatively, exon A might interact with proANP; the effect of PBA would then be explained by a PBA-induced change in conformation of exon A. Further experiments are needed to clearly define the protein-protein interactions between proANP and PAM.

Role of PAM in Shaping Atrial Secretory Vesicles

Mutation or deletion of the calcium-binding N-terminal proANP results in deformation of atrial secretory vesicles.13 One explanation is that coexpressed N-terminal–mutated or N-terminal–deleted proANP would be unable to precipitate with endogenous proANP, thus strongly diminishing the interaction between proANP and a hypothetical integral vesicle membrane protein.13 As a consequence, packaging of the vesicle core by TGN membrane would be deranged, and the vesicle would be deformed. The new results support this hypothesis. Figure 7A shows that a 3-day treatment with PBA causes a striking increase in deformed secretory vesicles, suggesting that PBA, by perturbing the interaction between PAM-1 and proANP, deranges the ordered packaging of secretory vesicles and contributes to the shap-
leukemia cells. To what degree integral vesicle transmembrane proteins may interact with PHM products, BAR (Bin/Amphiphysin/Rvs) domain proteins, or tyrosine phosphatases to shape the secretory vesicles remains open to investigation.

Is PAM Involved in Vesicle Docking?

Our previous study showed that deletion or mutation of N-terminal proANP not only deformed the secretory vesicles but also strongly diminished docking (illustrated in Figure 8B). Vesicle docking was thought to result from an interaction of docking receptors with plasma membrane docking proteins, and the lack of docking was attributed to suppression of recruitment of docking receptors. To test whether PAM could represent this docking receptor, vesicles were analyzed after 2 to 4 days of PBA treatment. This clearly reduced recruitment of PAM-1 and deformed a majority of secretory vesicles but had no effect on vesicle docking (Figure 7B), suggesting that PAM-1 is not likely to be involved in vesicle docking. So far, PAM-2 also is an unlikely candidate, because recruitment of PAM-2 is not affected by mutations of N-terminal proANP that are associated with decreased docking. A recent study on atrial secretory vesicle associated proteins should open the way to exploring several new candidates.

Conclusion

PAM proteins have long been suspected to play a role in the secretory pathway of the heart atrium and to be recruited to secretory vesicles via their C-terminal domain (also see the introduction). In this study, we show that PAM-1 is recruited to the atrial vesicles via an additional mechanism, most likely via interactions of its luminal domain with the calcium-binding domain of proANP. PAM-1 is involved in shaping the ANP-secretory vesicles, because the lack of recruitment of PAM-1 is associated with a high proportion of deformed ANP-secretory vesicles. The results further suggest that proteins distinct from PAM are involved in docking.

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