Localization of Atrial Natriuretic Peptide in Caveolae of In Situ Atrial Myocytes

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Abstract The plasma membrane–associated non–clathrin-coated vesicles called caveolae are multifunctional organelles thought to be implicated in the sequestration and transport of small molecules (potocytosis) as well as in the binding of Ca^{2+} ions, signal transduction, and processing of hormonal and mechanosensitive signals. We have previously suggested that the apparent contiguity of caveolar and atrial granule membranes observed in electron micrographs of in situ mouse atrial myocytes might reflect externalization of atrial natriuretic peptide through caveolae. Using Tokuyasu’s classic technique, we now show by immunoelectron microscopy of glutaraldehyde-fixed and cryosectioned mouse and rat atria that antibody against atrial natriuretic peptide prohormone is present within caveolae of in situ atrial myocytes. We confirm this intracaveolar localization by stereoimaging colloidal gold-labeled antibody to the prohormone in electron micrographs of glutaraldehyde/osmium tetroxide–fixed positively stained atrial thin sections. Because profiles of caveolae were rarely immunolabeled with antibody against atrial peptide unless there was a profile of an immunolabeled atrial granule nearby in the subjacent cytoplasm, we concluded that the intracaveolar hormone was derived predominantly from a direct interaction of atrial granules with caveolae. Perturbations that markedly increase the rate of natriuretic peptide secretion via the regulated pathway, including atrial stretch, contractions, and increased external Ca^{2+} concentration, failed to alter caveolar immunostaining. These results suggest that atrial peptide can pass from atrial granules into caveolae by transiently open pathways between the interiors of granules and caveolae. The results are interpreted as suggesting the presence of a second pathway for externalization of atrial natriuretic peptide through caveolae in addition to the classic pathway for regulated atrial peptide secretion at noncaveolar plasmalemma. (Circ Res. 1994;75:949-954.)

Key Words • caveolae • atrial natriuretic peptide • stretch dependence • cardiac membranes • signal transduction

Caveolae are flask-shaped plasmalemmal invaginations to present in all types of muscle cells and in many other mammalian cell types. These plasma membrane–associated vesicles, long identified with transcytosis in polarized cells, have recently emerged as multifunctional organelles that act in diverse cell types as sites for concentration and subsequent internalization of small molecules, a presumably caveola-specific process termed “potocytosis” by Anderson et al.6 Caveolae also function as binding sites for Ca^{2+} ions4 and molecules implicated in signal transduction4 and as sites for processing hormonal and mechanosensitive signals for the cell.6 Caveolae occupy 4% to 6% of the plasmalemmal area of mammalian ventricular myocytes,7,8 but their functions in ventricular and atrial myocytes are at present incompletely defined. We have recently related cardiac caveolae to stretch by showing that stretching rat atria with a physiological distending pressure of 5.1 mm Hg rapidly and reversibly permeabilizes the caveolae of atrial endocardium and of atrial myocytes to macromolecules.9 The reversible activation of atrial myocyte caveolar permeability by stretch prompted us to examine whether caveolae are implicated in a second stretch-activated property of these myocytes, atrial natriuretic peptide (ANP) secretion.10,11 We had previously suggested such a relation, based on transmission electron micrographs showing a striking structural contiguity of the membranes of plasmalemmal caveolae and the membranes of subplasmalemmal atrial granules in mouse atrial myocytes in situ.12 In the present study, we show by immunoelectron microscopy that caveolae of in situ mouse and rat atrial myocytes contain ANP, which probably enters caveolae by interaction with atrial secretory granules. Because immunostaining of intracaveolar ANP was unaltered by perturbations that critically change the rate of ANP secretion via the regulated pathway—atrial contractions,10,11 stretch,12,13 changes in [Ca^{2+}]i—10,13,14—we suggest that the intracaveolar hormone may indicate the existence of a second pathway for the externalization of atrial peptides by atrial myocytes.

Materials and Methods

The ultrastructural studies were carried out on two preparations: (1) right atrial appendages excised with fine scissors after thoracotomy from ether-anesthetized 20- to 27-g BALB/c mice, as previously described,12 and (2) in vitro preparations of combined right and left atria9,11 excised under ether anesthesia from 250- to 300-g female Sprague-Dawley rats. The rat atrial preparations were either unstretched or stretched by a physiological distending pressure of 5.1 mm Hg in the apparatus described by Page et al.9,11

The mouse atria were used for conventional electron microscopy of positively stained thin sections or for immunoelectron microscopy. For conventional ultrastructural studies on mouse right atrial appendages, the tissue was rinsed in modified Krebs-Henseleit (KH) solution (see below), fixed with sodium cacodylate–buffered 3% glutaraldehyde (pH 7.4), postfixed in 0.8% OsO4 at 0°C, embedded in Epon, thin-
sectioned, stained with uranyl acetate and lead citrate, and photographed at an original magnification of ×25 000 in a Hitachi 600 transmission electron microscope exactly as previously described.¹²

For immunostaining of mouse and rat atria, we used 5- or 10-nm colloidal gold–labeled goat anti-rabbit secondary antibody against primary antibody made in rabbit against amino terminal amino acid sequences 1 to 28 of rat α-ANP, an antibody that detects the prohormone.

We used the classic fixation and cryoultramicrotomy method of Tokuyasu¹³ to prepare specimens of atria for immunoelectron microscopy. Tokuyasu’s method was chosen because it combines preservation of well-defined membrane profiles with retention of the immunoreactivity of antigenic sites. For this purpose, atrial preparations were fixed on ice with 0.25% glutaraldehyde for 30 minutes, infiltrated with 2.3 mmol/L sucrose in phosphate-buffered saline (PBS) for 60 to 90 minutes, frozen on micromotive chucks in liquid nitrogen, and sectioned at section thickness settings of 80 to 90 nm with an FC-4D cryoultramicrotome (Reichert) at specimen knife temperatures of ~90°C to ~−9°C. Sucrose was then replaced by a drop of sucrose/PBS and a platinum loop. The loop with the section on it was touched to carbon-coated grids glow-discharged in a Denton DV502 vacuum apparatus. The grids were then floated (section side down) onto PBS cooled on ice. The grids were then labeled at 21°C as described by Tokuyasu by sequential exposure to PBS (5 minutes), 50 mmol/L glycine/PBS (5 minutes), PBS (5 minutes), 100% goat serum/PBS (15 minutes), primary ANP antibody 1:200 in 10% goat serum/ PBS (60 minutes), four rinses in PBS (5 minutes per rinse), 100% goat serum (15 minutes), secondary antibody labeled with 5- or 10-nm colloidal gold at a dilution of 1:50 in 10% goat serum/PBS (60 minutes), four rinses in PBS (5 minutes per rinse), four rinses in distilled water (total of 5 minutes) to remove phosphate ions, 4% uranyl acetate plus 0.3 mol/L oxalic acid neutralized with 10% NH₄OH (10 minutes), distilled water (1 second), 4% aqueous uranyl acetate (10 minutes), and distilled water (1 second), followed by three drops of methyl cellulose on ice for 1 second each (by using a 2% solution of 25 rpm methyl cellulose, 1.8 mL of methyl cellulose having been mixed with 0.2 mL of 4% uranyl acetate for use). After thus staining the sections with uranyl acetate, they were dried for examination by electron microscopy in a Hitachi 600 transmission electron microscope.

Isolated combined right and left atrial preparations (converted into a single chamber by perforating the interatrial septums⁹,¹¹) were briefly preincubated with modified KH solution containing 1.4 mmol/L [Ca²⁺], to seal gap junctions at the cut surfaces⁹,¹¹ and then studied by one of three protocols before fixation for immunoelectron microscopy by the method of Tokuyasu¹³ described above: For the first protocol, five noncontracting, unstretched atrial preparations were incubated for 5 minutes at 37°C in modified KH solution. [Ca²⁺] was reduced to 0.2 mmol/L, with 10 μmol/L ryanodine added to induce relaxations and to inhibit spontaneous contractile biorhythms. The second protocol was identical to the first, except that atria were stretched in the apparatus of Page et al.¹⁰,¹¹ by a distending pressure of 5.1 mm Hg. The third protocol was identical to the second protocol, except that [Ca²⁺] was raised from 0.2 to 1.4 mmol/L. At the end of the incubation period, rat atrial preparations were fixed with 0.25% glutaraldehyde for 30 minutes at 21°C for immunoelectron microscopic localization of ANP by Tokuyasu’s method. Primary antibody against ANP was omitted in control sections to test for nonspecific staining, whereas both these controls and the sections exposed to primary antibody against ANP were treated with secondary colloidal gold–labeled antibody. Primary rabbit polyclonal antibody against rat α-ANP was obtained from Peninsula Laboratories, Inc; secondary colloidal gold–labeled goat antirabbit IgG was obtained from Ted Pella, Inc.

A preparative technique different from the protocol of Tokuyasu¹³ was used to obtain electron micrographs in which the location of the colloidal gold–labeled secondary antibody in caveolae and atrial granules could be seen by stereoimaging. For this purpose, right atrial appendages from two rat hearts (sealed and preincubated for 10 minutes in control solution containing 0.2 mmol/L [Ca²⁺], and 0.01 mmol/L ryanodine) were fixed for 30 minutes at 20°C in 1% glutaraldehyde buffered to pH 7.3 with 150 mmol/L sodium cacodylate, rinsed three times for 10 minutes in sodium cacodylate buffer, and postfixed for 10 minutes at 4°C in 0.8% OsO₄ in sodium cacodylate buffer. After dehydration and embedding in Epon, sections ≈50 nm thick were cut by conventional ultramicrotomy¹² and picked up on nickel grids. The sections on the grids were rinsed 5 minutes with distilled water, etched for 15 minutes with 10% hydrogen peroxide, and rinsed for 5 minutes in PBS. The glutaraldehyde was quenched for 10 minutes with 50 mmol/L glycine in PBS, rinsed again in PBS for 5 minutes, blocked with 100% goat serum, and then stained with primary and secondary antibody as above and with uranyl acetate and lead citrate as previously described.¹³ Sections prepared in this way at 0° and +10° of tilt were photographed at an original magnification of ×30 000 by using the goniometer stage of a Hitachi 600 electron microscope to tilt the specimen by 10° for stereoimaging. Stereopairs of positive prints of the electron micrographs (printed at a magnification of ×30 000) were viewed at a magnification of ×2.25 with a Pocket Stereoscope (Ted Pella, Inc) to obtain a three-dimensional display of the distribution of immunogold in caveolae and atrial granules.

The composition of the modified KH solution used for scaling and rinsing mouse atria was (mmol/L) Na⁺153.4, Cl⁻ 161.3, K⁺ 5.92, Ca²⁺ 1.40, Mg²⁺ 0.56, and glucose 25, buffered to pH 7.3 with 2.0 mmol/L tris(hydroxymethyl)aminomethane maleate and 2.0 mmol/L N-acetylglycine.¹² The composition of the corresponding solution used for rat atrial preparations was (mmol/L) Na⁺ 130, Ca²⁺ 0.2, K⁺ 5.92, Mg²⁺ 0.56, Cl⁻ 137, HEPES 10, and glucose 25 (pH 7.3), Ca²⁺ being raised to 1.4 mmol/L for one group of experiments. The composition of PBS was (mmol/L) NaCl 150, NaHPO₄·H₂O 10, and NaH₂PO₄ 10 (pH 7.4).

Results

Fig 1a and 1b, transmission electron micrographs of conventionally fixed and stained thin sections of mouse atrium, illustrate how closely the limiting membrane of an atrial granule approaches the cytosolic surface of the overlying plasmalemmal caveolae. This previously noted configuration¹² suggested the speculation that the two membranes might interact to transfer ANP from atrial granules into the caveolae and then into the interstitial space.¹⁵ To investigate whether caveolae of in situ rodent atrial myocyte function as binding sites, storage sites, or pathways for ANP, we looked for intracaveolar ANP by immunoelectron microscopy.

Fig 1c is an electron micrograph of mouse atrial myocyte plasma membrane and subjacent cytoplasm. The micrograph was obtained from a spontaneously contracting atrium fixed and frozen immediately after excision at [Ca²⁺]₀ of 1.4 mmol/L. Ultrathin cryosections were immunogold-labeled for ANP. The atrial granules in Fig 1c are labeled at high density. Profiles of several plasmalemmal caveolae overlying the granules are also labeled. Mitochondria and cytoplasm contain only rare gold particles, as did controls incubated without primary antibody. On the basis of the positions of the caveolae and atrial granules in electron micrographs like Fig 1c, we consider it very unlikely that the presence of colloidal gold over caveolar profiles is an artifact.
caused by superposition of a labeled granule on one side of the section upon an unlabeled caveola on the other side of the section.

Fig 2a and 2b and the primary antibody-free control (Fig 2c) are electron micrographs from comparable in vitro experiments representative of five unstretched noncontracting rat atria incubated at 37°C with \([\text{Ca}^{2+}]_0=0.2\) mmol/L. Contractions were prevented by addition of 10 μmol/L ryanodine. Fig 3a and 3b (\([\text{Ca}^{2+}]_0=0.2\) mmol/L) and 3c and 3d (\([\text{Ca}^{2+}]_0=1.4\) mmol/L) are otherwise identical experiments, each performed in duplicate, in which atria were stretched by a physiological distending hydrostatic pressure of 5.1 mm Hg for 5 minutes, as described by Page et al.\(^9,11\)

Fig 2a and 2b (but not control, Fig 2c) show specific ANP immunostaining at low \([\text{Ca}^{2+}]_0\). Stretching two atria in a solution containing 0.2 mmol/L \([\text{Ca}^{2+}]_0\), a perturbation that increases the ANP secretory rate 3.6-fold,\(^11\) did not detectably alter ANP antibody immunostaining of caveolar profiles (Fig 3a). Raising \([\text{Ca}^{2+}]_0\), of two stretched atria for 5 minutes from 0.2 to 1.4 mmol/L, a procedure that inactivates secretion after an initial transient increase in secretory rate,\(^13\) also did not interfere with caveolar immunostaining (Fig 3c).

Figs 1c and 2a show immunogold labeling of Golgi cisternae and trans-Golgi network (TGN) in the cytoplasm underlying labeled atrial granules. Since we are
sional localization within caveolae of the colloidal gold on secondary antibody to proANP can be visualized by looking with a stereoviewer at the lower magnification stereopair prints of the pinwheel (Fig 4b and 4c). We explain the occasional finding of label overlying cytoplasm by the failure of the thin section to include the entire caveolar membrane profile, by membrane profiles damaged or tangentially sectioned in the microtome, or by the presence of vicinal atrial granules lying just outside the plane of section. The stereomicrographs (Fig 4b and 4c) support the conclusion from Figs 1 through 3 that proANP is present intracaveolarly and provide a three-dimensional view of the label on the concave internal membrane surface of the caveolar cavity.

**Discussion**

The most probable interpretation of these results is that ANP can pass from atrial granules into caveolae via transiently open pathways between the interiors of atrial granules and caveolae. The results do not, however, prove that caveolae are the only route, or necessarily the main route, by which atrial granules normally discharge their contents into the interstitial spaces, since we and others have published electron micrographs of thin-sectioned atria and of freeze-fractured atria showing fusion of atrial granule membranes with noncaveolar plasma membrane, indicative of regulated ANP secretion by exocytosis. Instead, the intracaveolar ANP described in the present study may arrive there via a different pathway for externalizing ANP. The externalized ANP in caveolae should be responsive to caveolae-specific signals and regulatory mechanisms, which differ in major respects from those for classic exocytosis. Alternatively, or in addition, bound intracaveolar ANP might have entered caveolae from the interstitial spaces, e.g., as a feedback mechanism by which atrial myocytes could monitor and react to the prevailing interstitial ANP concentration produced by their own secretion and by that of neighboring myocytes. But because we find that it is unusual for profiles of caveolae to be labeled with antibody against ANP unless there is a profile of a labeled atrial granule nearby in the subjacent cytoplasm, we conclude that intracaveolar ANP derives predominantly from an interaction of atrial granules with caveolae.

That three perturbations, each of which greatly increases the rate of ANP secretion via the regulated pathway, seem not to alter caveolar immunolabeling by antibodies to ANP raises the possibility that intracaveolar ANP reflects a second pathway for ANP externalization, a pathway that operates in parallel with the regulated pathway and not necessarily synchronously with it. This interpretation would not violate the constraint that caveolae should not be simultaneously open to the cytosol and to the interstitial space. A model in which high-affinity intracaveolar ANP-binding sites on the caveolar membrane are in equilibrium with a concentration of intracaveolar free ANP that is small compared with that in the regulated pathway of ANP secretion would be consistent with our observations and with either or both of two extracaveolar sources of ANP: (1) ANP taken up from the interstitial spaces when caveolae are open to the interstitium and/or (2) ANP entering caveolae from the cytoplasmic surface of the
caveolar membrane and derived from an interaction between caveolae and atrial granules.

The interpretation of intracaveolar ANP as evidence for a secondary pathway for externalizing ANP requires qualification. First, although stretch seems not to trigger entry into it, we do not know what does. Second, our observations so far in no way rule out the possibility that, under some as yet unidentified condition(s), the caveolar pathway might become a major or even the dominant pathway for ANP secretion in rodent atria. The role of caveolae in the constitutive secretion of ANP also needs to be examined, especially for ventricular myocytes of adult rats, in which ANP secretion is normally predominantly constitutive.

Caveolae contain bound Ca^{2+}, and they cycle between states open to and closed off from the interstitial space. When atrial myocyte caveolae are closed to the interstitial space, intracaveolar Ca^{2+} content and ionized [Ca^{2+}] are probably controlled by the cardiac caveolar Ca^{2+}-pump ATPase and a caveolae-associated isoform of inositol triphosphate receptor. Together, these two Ca^{2+}-transporting proteins may also determine [Ca^{2+}] in the subplasmalemmal cytosolic microdomains underlying caveolae. In noncardiac cell types, these microdomains are sites of multiple caveolae-associated signal-transducing molecules. In this regard, it is of particular interest that a 67-kDa Ca^{2+}-binding protein, p67, which is localized at the sarcolemma of mammalian atrial myocytes, has recently been described by Iida et al. It is also noteworthy that if, while closed off from the interstitial space, the interiors of caveolae become transiently continuous with the interiors of atrial granules (as suggested by the results of our
experiments), the intracaveolar compartment would be transiently exposed to the high Ca\textsuperscript{2+} content and acid pH prevailing inside these granules.\textsuperscript{22}

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