Rat Atrial Myocyte Plasmalemmal Caveolae In Situ

Reversible Experimental Increases in Caveolar Size and in Surface Density of Caveolar Necks

Leszek Kordylewski, Gwendolyn E. Goings, Ernest Page

The structure, size, and surface density of the conspicuous flask-shaped structures called caveolae that are located under the plasma membrane of cardiac myocytes in intact rat atria were studied by electron microscopy after physiological perturbations designed to examine whether caveolae and/or their necks are fixed or mobile and whether the caveolar lumen is always open or can close off from the interstitial space. We showed that, in stretched and unstretched atria, horseradish peroxidase could enter or be washed out of caveolae at 37°, 18°, and 4°C, but this finding does not rule out that caveolae and/or their necks can cycle rapidly between states closed and open to the interstitial space. Electron microscopy of thin sections revealed that exposure of atria at 37° or 18°C to physiological salt solutions made hypertonic by adding 150 mM sucrose or mannitol resulted in a striking enlargement of caveolar profiles within 1 to 5 minutes. Caveolar enlargement was rapidly reversible on return to control saline. After freeze fracture of atria exposed to these hypertonic solutions, quantitative analysis of electron micrographs of the fracture faces revealed statistically significant increases in cross-sectional diameter of cross-fractured caveolar necks and in mean number of caveolar necks penetrating per unit area of plasmalemmal fracture face. These results suggest that atrial myocyte caveolae are dynamic structures whose necks may be reversibly inserted into and withdrawn from the plasmalemma, possibly (but not necessarily) corresponding to states in which caveolae are, respectively, open and closed to the interstitial spaces. (*Circulation Research* 1993;73:135-146)

Key Words • caveolae • plasma membrane ultrastructure • caveolar neck • nonclathrin-coated vesicles

Caveolae are conspicuous flask-shaped structures located under the cardiac plasma membrane into which they insert via narrow necks.[1-5] A recent review[6] restates the current consensus that, like the caveolae of skeletal muscle[1,7] and unlike clathrin-coated pits and vesicles, the caveolae of mammalian heart muscle cells in situ remain fixed in place under the plasma membrane and that the caveolae (and the caveolar necks that are inserted into and pass through the cardiac myocyte plasma membrane) do not migrate toward the interior of the myocyte.[6] Although the functions of the caveolae associated with the plasma membrane of heart muscle cells remain unidentified, these structures have been provisionally included in a category of nonmigratory plasmalemmal vesicles that, when visualized by electron microscopy of thin sections from conventionally fixed and stained tissues, lack the prominent protein coats on their cytoplasmic surfaces that are a characteristic of clathrin-coated pits and vesicles. In this respect, the caveolae of cardiac myo-cytes resemble apparently uncoated plasma membrane–associated vesicles of vascular endothelial cells that are implicated in transcytosis.[8] They are also similar to apparently uncoated vesicles of atrial endocardial endothelium, which take up and presumably transport macromolecules when atria are stretched.[9] Caveolae have been isolated from nonmuscle cells by Rothberg et al,[10] who found that they contain a filamentous cytoplasmic surface protein that has a 22-kD subunit, "caveolin." This is an apparently unique protein of 178 amino acids containing an unusual 40–amino acid hydrophobic region near the C terminus, a sequence that may serve to anchor the protein to the caveolar membrane.[11] Antigens made from caveola-like vesicular structures engaged in transcytosis have been produced by Sztul et al.[12] Rothberg et al[13] concluded from experiments on plasmalemma-associated caveolae in an epithelial cell line derived from monkey kidney that caveolae cycle between two states: a state in which their interior is open and accessible to the extracellular medium and a second state in which the caveolar interior is closed off from and inaccessible to the extracellular medium. They further suggested that, in the closed state, the caveolar lumen might become acidified and that folate bound to folate receptors in the caveolar membrane is translocated from the luminal to the cytoplasmic face of the membrane by specific transporters in the caveolar mem-

brane. Recently, Fujimoto et al[14] have found that caveolae of endothelium, smooth muscle cells, and keratinocytes could be specifically immunostained on their cytoplasmic surface by monoclonal antibodies against a type I inositol-1,4,5-trisphosphate receptor–like protein.

In the present study, we have used a combination of physiological and ultrastructural methods to perform in vitro experiments on caveolae of rat atrial myocytes in intact noncontracting atria from adult rats.[15-17] The experiments examine whether and under what conditions atrial myocyte caveolae may be open or closed with respect to the interstitial space and whether and under what conditions caveolae and caveolar necks can undergo changes in size and in surface density (number per unit plasma membrane area). The results show that the caveolae of atrial myocytes in situ are much more dynamic structures than envisioned by the present consensus model, that they are capable of undergoing rapid and rapidly reversible enlargement in response to an increase in extracellular osmolarity, and that both the surface density (number per unit area) of caveolar necks inserted into the lipid bilayer of the plasma membrane and the cross-sectional diameters of these necks increase concomitantly with caveolar enlargement. The results are interpreted to support a model in which the lumina of caveolae in cardiac myocytes can be reversibly closed off from the interstitial space by withdrawal of the caveolar necks from the lipid bilayer of the plasma membrane, by caveolar neck closure, or by a combination of these mechanisms.

Materials and Methods

Experimental Design

Female Sprague-Dawley rats aged 3 to 4 months were anesthetized with ether. Their hearts were excised, rinsed, sealed at the cut edges with solutions containing 1.4 mM $[Ca^{2+}]$, and used to make in vitro preparations of combined left and right atrial chambers by perforating the interatrial septum as previously described Page et al.[15,18] The preparations, which were either stretched by a constant distending pressure of 5.1 mm Hg or left unstretched, were incubated in a thermostated oxygenated solution using the apparatus[15] diagramed schematically by Page et al.[9] All preparations were rendered noncontracting by including ryanodine (10 μM), both during preincubation and in the experimental incubating solutions, which (unless otherwise noted) had $[Ca^{2+}]_o$ of 0.2 mM. The experiments were carried out at 37°C, except for selected experiments at 18° or 4°C as indicated in the text. For experiments in which caveolae of atrial myocytes in situ were loaded with the fluid-phase endocytosis probe horseradish peroxidase (HRP), the preparation was exposed to HRP at 18°C on both the endocardial and epicardial surfaces after stretching the atria by the distending hydrostatic pressure of a 7.0-cm column of saline (5.1 mm Hg).[9] This procedure reliably fills both the caveolae of the atrial myocytes and the interstitial spaces between myocytes with HRP, as determined in electron micrographs of thin sections by histochemical staining for HRP.[9] At the end of each experiment, the preparations were fixed in the stretched or unstretched state by immersion in buffered glutaraldehyde, postfixed with OsO_4, dehydrated, embedded, thin-sectioned for electron micros-

copy, and either stained for peroxidase[9] or stained conventionally for ultrastructural studies.[19] Other specimens were fixed with buffered glutaraldehyde only and subjected to freeze fracture as described by Kordylewski et al.[20] For experiments in which osmolarity was the crucial variable, the osmolarity of the primary glutaraldehyde-containing fixative was made equal to that of the experimental solution used immediately preceding fixation. That the osmolarities were equal was proved by measurements of the freezing point depression with an osmometer (Fiske Associates, Uxbridge, Mass).

Since HRP predictably and uniformly enters caveolae of in situ atrial myocytes only when presented at the endocardial surface of stretched atria,[9] all experiments testing for uptake of HRP by myocardial cells or involving preloading of caveolae with HRP required initial stretching of the atria for this purpose. Subsequent tests for unloading of HRP from caveolae were then carried out in the unstretched state because, as explained by Page et al,[9] the design of the apparatus precluded the use of stretched preparations since it did not permit changing the solution in the 7.0-cm column of fluid above the endocardium. For tests of HRP unloading, the preparation was therefore removed from the column after completion of loading in the stretched state at 18°C and immersed without stretching in an oxygenated, thermostated, and gently stirred beaker containing the HRP-free unloading solution at 37° or 4°C.

For experiments using the freeze-fracture technique to determine the effect produced on the surface density of caveolar necks or the diameters of caveolar necks by incubation in a solution made hyperosmolar (hypertonic) by adding sucrose (to a final concentration of 150 mM) to the control solution, the right atria were cut off and divided into two pieces. Their gap junctions at the cut surfaces were sealed in 1.4 mM Ca^{2+}-containing solution. One piece was incubated for 5 minutes in the hyperosmolar solution; the other was incubated in sucrose-free control solution. (Both solutions contained 10 μM ryanodine and 0.2 mM $[Ca^{2+}]$, as usual.) The experimental half was then fixed in a glutaraldehyde-containing fixative made hypertonic with 150 mM sucrose (and therefore of the same hypertonic osmolarity as that which prevailed during the preceding incubation). The control piece was similarly fixed in a glutaraldehyde-containing medium that was sucrose free and isosmolar with the control incubation medium. For experiments using the freeze-fracture technique to examine the reversibility of the effects of hyperosmolar solutions, atria were cut into two pieces, which were then successively incubated for 5 minutes (after sealing and before fixation) either (1) in hypertonic sucrose-containing medium followed by immediate fixation at the same osmolarity or (2) in hypertonic sucrose-containing medium followed by a second 5-minute incubation in sucrose-free control medium, followed in turn by fixation at control osmolarity.

Solutions

The composition of the isotonic solution for experiments on the open or closed state of caveolae in intact atria was (mM) Na^+, 130; Ca^{2+}, 0.2; K^+, 5.92; Mg^{2+}, 0.56; Cl^-, 137; HEPES, 10; and glucose, 25. The solution was adjusted to pH 7.4 and was oxygenated during the experiment by passing water vapor–saturated 100%

O_2 over its surface. The composition of the primary fixative (isosmolar by osmometry with the isotonic control solution) was (mM) sodium cacodylate, 25; KCl, 5.9; $CaCl_2$, 0.8; and glutaraldehyde, nominally 200 (actually 2%), pH adjusted to 7.3 to 7.4.

For experiments testing the effect of raising external osmolarity on caveolar size, caveolar neck size, and surface density of caveolar necks, the osmolarity was raised by addition of either 150 mM sucrose or 150 mM mannitol to a control solution that was identical to the above isotonic solution except that its total Na^+ and Cl^- concentrations were, respectively, 98 mM and 105 mM. Consequently, the osmolarity of the sucrose- or mannitol-free control was approximately 58 mOsm/L smaller than that of the above isotonic control. When the experimental solution was made hypertonic with 150 mM sucrose or mannitol, the primary glutaraldehyde fixative was made isosmolar with the experimental solution by adding 150 mM sucrose or mannitol. Postfixation with OsO_4, embedding, ultramicrotomy, staining with salts of lead and/or uranium, staining for HRP, and electron microscopy were as described by Page et al[15,19] for intact atria. Intact atria fixed with isosmolar fixative did not differ discernibly from atria fixed with conventional fixative containing 3% glutaraldehyde, a concentration that gives a somewhat higher yield of uniformly satisfactorily fixed specimens. Nevertheless, we thought it necessary, because of the experimental question addressed in these experiments, to use a fixative that had the same osmolarity as that used in the experimental condition prevailing just before fixation for all experiments, including those assayed by image processing of electron micrographs of freeze-fractured preparations. The sucrose and mannitol used in these experiments were American Chemical Society reagent grade. These agents, as well as 8-bromo adenosine 3':5'-cyclic monophosphate, *N*-methyl-D-aspartic acid (NMDA), sodium nitroprusside, ionomycin, superoxide dismutase, catalase, propranolol, norepinephrine, caffeine, and bumetanide were obtained from the Sigma Chemical Co, St Louis, Mo.

Image Processing

The surface density of caveolar necks in the plasma membrane was determined by photographing replicas of fracture faces derived from the half atrium incubated in control solution and from the other half atrium incubated in hypertonic solution. Specimens were photographed in a Hitachi H-600 electron microscope at original magnifications of ×10 000. Photographic negatives of replicas of control hemiatria and of the hemiatria exposed to hyperosmolar solution were coded, randomized, and processed by the image processing system, with the observer blinded with respect to the origin of the negatives. The system consisted of a Sony Trinitron monitor interfaced with a Zeos 386 computer using Olympus C-2 image analyzer software. Digitized images were projected onto a monitor screen, and data were received as the number of caveolar necks per 16 μm^2 of plasmalemmal fracture face. The number of areas per replica (five areas) and the number of replicas per hemiatrium (six to eight replicas) were chosen so that the mean number of caveolar necks per 16 μm^2 fracture face (averaged over all fracture faces for that hemiatrium) converged toward a constant value. This value was expressed as mean±SEM (averaged over the total number of 16-μm^2 areas measured).

The same imaging system was used to quantify changes in the mean cross-sectional diameters of cross-fractured caveolar necks as measured in the plasmalemmal fracture faces of atrial myocytes when unstretched hemiatria from the same atrium were exposed for 5 minutes at 37°C to control solution or to control solution made hypertonic by addition of 150 mM sucrose. These measurements were made on the same atria and photographic negatives previously used to measure changes in caveolar neck surface densities. To measure caveolar neck diameters, the negative film from the electron microscope camera was placed on a light box. A television camera with an Olympus lens mounted over the light box was used to project the profiles of cross-fractured caveolar necks onto the screen of the monitor, thereby enlarging the original photographic image approximately 15-fold. In this way, from 1 to 30 caveolar necks could be seen, the number depending on caveolar neck surface density in that part of the fracture face. A total of 4500 caveolar necks were thus measured (50 necks × 5 micrographs × 3 replicas × 2 atrial halves × 3 rats). The system was calibrated using the film of a calibration grid photographed at a magnification of ×10 000 in the electron microscope. Measurements of neck cross-sectional diameter, obtained on the screen by fitting a circle of variable size to the projected profiles of caveolar necks using the optical mouse, were stored in the computer memory. Care was taken to image-process only level well-preserved areas of the plasmalemmal fracture face. Since there were many more caveolar neck profiles in each micrograph than could reasonably be measured, the maximal number per micrograph was arbitrarily limited to 50. Neck diameters were measured only if the stumps were round, had a distinct well-defined rim that was not obscured by shadowing metal, and were not otherwise deformed.

Results

Caveolae of In Situ Atrial Myocytes Are Normally (at Least Transiently) Open to the Interstitial Space

Longitudinally oriented thin sections of atrial myocytes from atria fixed with glutaraldehyde, postfixed with OsO_4, and stained with uranyl acetate and lead citrate yield classical profiles of caveolae and caveolar necks (Fig 1). As previously noted by multiple authors (see review by Severs[6]), a caveolar neck may appear to be the outlet for a single caveolar profile or for multiple caveolar profiles (Fig 2). In some profiles, the outlet of the caveolar neck may seem closed (Fig 1). In most profiles, the necks look open (Fig 1), but the interiors of the caveolae are separated from the interstitial space between myocytes at their outlets to the interstitium by a thin diaphragm (Fig 3). In a single exploratory experiment, we compared 223 consecutive caveolar profiles from a preparation stretched by a distending pressure of 5.1 mm Hg with 258 consecutive caveolar profiles from an unstretched atrial preparation (0 mm Hg distending pressure). Measurements (made on nonserial sections) were arbitrarily limited to profiles in which only one caveola was associated with one caveolar neck. In this sample, 33% of caveolar profiles from stretched atria and 23% of caveolar profiles from unstretched atria

FIG 1.   *Electron micrograph of a longitudinally oriented thin section through the plasma membrane of an unstretched noncontracting rat atrial preparation incubated in control solution at 37°C. Note multiple caveolar profiles beneath the sarcolemma, either apparently open to the interstitial space (IS) via a caveolar neck (curved arrow) or apparently unconnected with the overlying sarcolemmal membrane (arrowhead). IS contains collagen and basal lamina (arrow).*

appeared open, as judged by the continuity of the caveolar lumen with a neck that opened out into the interstitial space.

The impression that caveolae are closed at the neck by apposition of their sides proved, however, to be wrong. As previously shown by others,[6] we found that ultrathin serial sections of atria invariably revealed that such apparently closed profiles were open in at least one of the three consecutive sections required to pass through the entire caveola. Moreover, as we have previously noted,[3] serial sections or single sections of atria just below and parallel to the plane of the membrane yielded characteristic pinwheel or cloverleaf configurations in which multiple caveolae were seen to surround a single neck (Fig 4).

A convincing alternative method of evaluating whether the caveolae of atrial myocytes are open or closed to the interstitial space is to load them with the electron-opaque fluid-phase probe HRP. In a previous publication, we showed that HRP enters caveolae of intact atria[9] at 37°C (n=8) and 18°C (n=2), provided HRP is introduced on the endocardial surface of the atrium. HRP does not undergo endocytosis in heart muscle cells of intact atria,[9] although it is internalized by primary cultures of myocytes made from such atria.[21] Thus, when noncontracting rat atria were stretched at 18°C in control solution containing 0.2 mM $[Ca^{2+}]_o$ and 10 μM ryanodine, the plasmalemmal caveolae of their
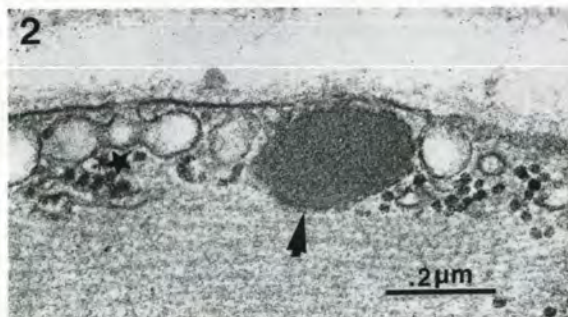

FIG 2.   *Electron micrograph of atrial sample, as described in Fig 1, showing a caveolar neck (star) apparently connecting at least two caveolar profiles with the interstitial space. Atrial granule (arrowhead) lies between single caveolar profiles.*

FIG 3.   *Electron micrograph showing caveolar profile apparently separated from the interstitial space by a thin diaphragm (arrowhead).*

cardiac myocytes became rapidly and reliably loaded with HRP (molecular mass, approximately 40 kD).[9] At 18°C, HRP was seen to opacify the interstitial space; therefore, the concentration gradient favors the diffusion of HRP through the caveolar necks into the interior of the caveolae (Fig 5). We have confirmed this finding in five additional preparations as part of the present study. These influx experiments clearly demonstrate that the solution in caveolar necks and caveolae equilibrates with that in the interstitial space and that the diaphragms across the caveolar outlets do not filter out HRP. In the present study, we show that the outflux of HRP from HRP-preloaded caveolae into HRP-free solution can then be demonstrated by cutting the stretched preparation from the cannula, incubating it in the unstretched state in an HRP-free solution, and fixing the tissue for electron microscopic identification of any HRP not washed out. Fig 6 shows the result of experiments of two different atrial preparations in which HRP inside caveolae preloaded at 18°C was successfully washed out into control solution at 37°C (for 1 minute) and at 4°C (for 30 minutes). At both temperatures (and also at 18°C), the probe was readily washed out, indicating that caveolae and their necks were open to the interstitial space and suggesting that opening of caveolae to permit HRP efflux does not require an input of metabolic energy.

We performed a series of exploratory experiments at 37°C to determine whether selected physiological or pharmacological perturbations could prevent the filling of myocyte caveolae with HRP or interfere with the washout of HRP from caveolae previously filled with HRP. The following single experiments did not affect entry of HRP into or loss of HRP from caveolae of atrial myocytes: (1) incubation with control solution containing 30 μM NMDA, (2) incubation with 2.0 μM sodium nitroprusside, (3) raising $[Ca^{2+}]_i$ with 1.0 μM ionomycin (in dimethyl sulfoxide) in the presence of 0.2 mM $[Ca^{2+}]_o$, (4) α_1-adrenergic stimulation with 5 μM norepinephrine in the presence of 1 μM propranolol, superoxide dismutase, and catalase, and (5) elevation of intracellular cAMP concentration with 10 μM caffeine.

Hyperosmolar Solutions Cause Reversible Enlargement of Caveolae

Another approach to identifying a subpopulation of caveolae that are closed off from the interstitial space is to incubate atria very briefly in a hyperosmolar (hypertonic) solution of sucrose. Assuming that water can
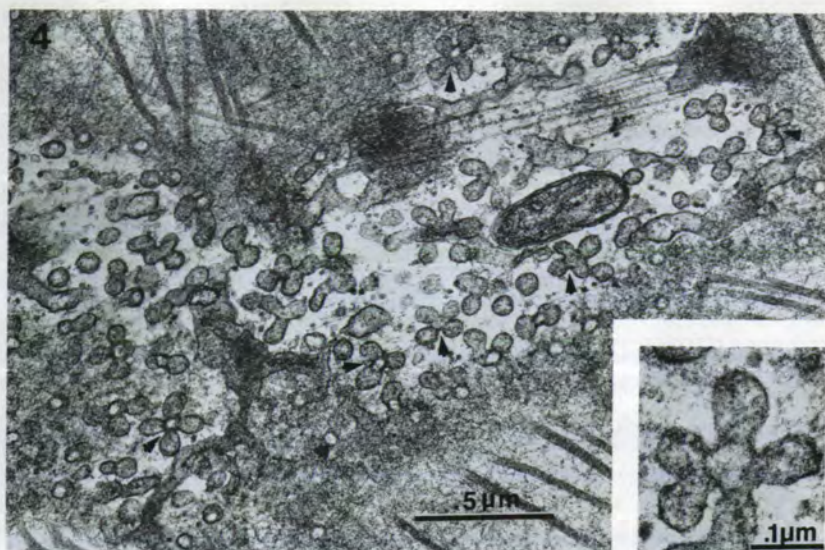
FIG 4. *Electron micrograph of atrial sample, as described in Fig 1, with section oriented to pass approximately tangentially to and just beneath the plasma membrane. Note multiple pinwheel or cloverleaf configuration of caveolae (arrowheads). Inset shows a typical pinwheel with five caveolar profiles oriented around one caveolar neck.*

diffuse through caveolar membranes, caveolae that are closed should initially undergo osmotic shrinkage, whereas open caveolae should remain unaffected. However, when we performed this experiment, we found no evidence of caveolar shrinkage. Instead of shrinking, the caveolar profiles became strikingly larger.

Experiments were therefore performed to examine the effect of 150 mM sucrose on caveolae of unstretched atria from five rats and of stretched atria from two rats, as well as on the reversibility of this effect on un-

stretched atria from two rats, and the effect of 150 mM mannitol on unstretched atria from one rat.

Fig 7a is an electron micrograph of plasmalemmal caveolae in a thin-sectioned in situ heart muscle cell from an unstretched atrial preparation incubated for 5 minutes at 37°C in a solution made hyperosmolar by adding 150 mM sucrose to the (slightly hypotonic) control incubation medium described in "Materials and Methods." The preparation was then fixed in a glutaraldehyde fixative containing 150 mM sucrose. This fixative had the same osmolarity as the hypertonic incubation medium, as determined by measuring the depression of the freezing point. The figure shows that,
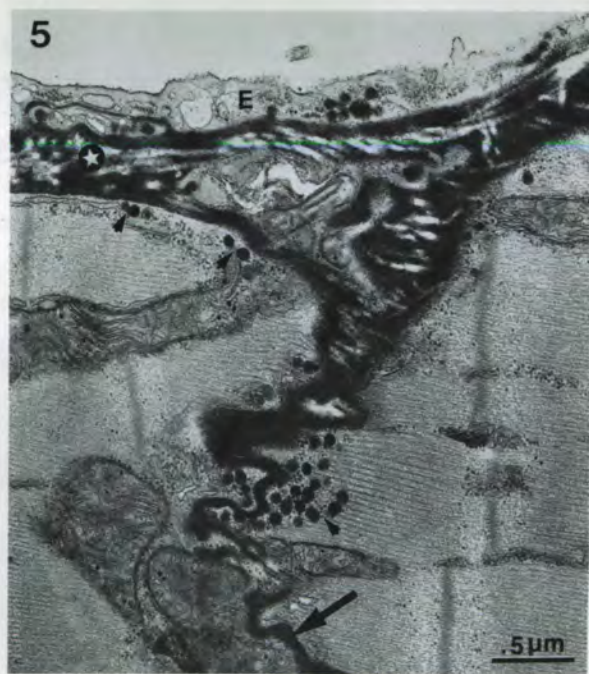

FIG 5. *Electron micrograph of stretched atrial preparation incubated at 18°C with horseradish peroxidase (HRP), stained histochemically for HRP, and counterstained with lead citrate. From top downward note endocardial endothelial cell (E) with HRP-containing vesicular profiles, subendocardial space (star) heavily stained with HRP, interstitial space between atrial myocytes (arrow) that is opacified with HRP, and atrial myocyte caveolae (arrowheads) filled with HRP.*
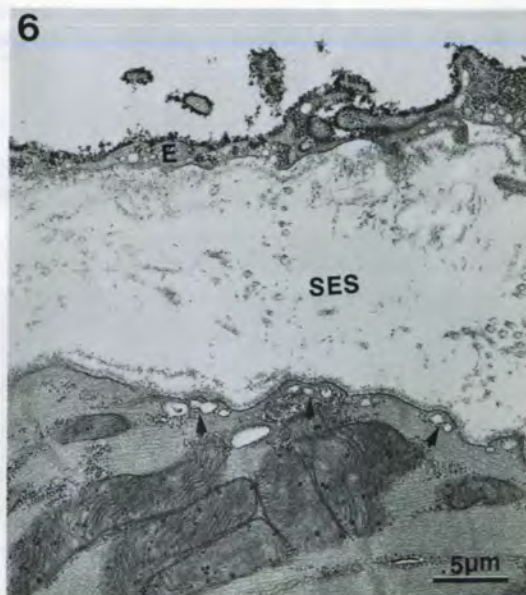

FIG 6. *Electron micrograph of atrial preparation loaded with horseradish peroxidase (HRP) at 18°C as documented for Fig 5 and washed out for 1 minute at 37°C in HRP-free control solution. Note that caveolae of atrial myocytes (arrowheads), as well as the subendocardial space (SES), are free of HRP, whereas HRP is still present on the surface and in the vesicles of the endocardial endothelial cells (E).*
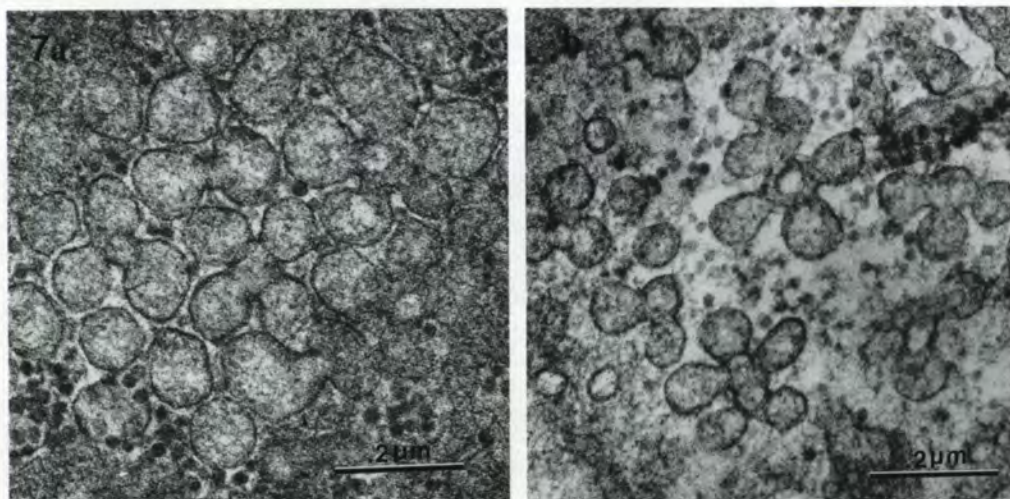
FIG 7.   *Electron micrographs of atrial preparation. The unstretched half atrium (panel a) was incubated for 5 minutes at 37°C in control solution made hypertonic by adding sucrose to a final concentration of 150 mM and then fixed at the same osmolarity in glutaraldehyde containing 150 mM sucrose. Section passes just below and parallel to plasma membrane. Note pinwheel configuration of caveolae showing that caveolar profiles are markedly enlarged by comparison to the caveolar profiles in the control half atrium (panel b), which was incubated and fixed in control (sucrose-free) medium.*

by comparison with caveolar profiles of atrial myocytes from preparations incubated and fixed at control external osmolarity (Fig 7b), the caveolar profiles of atria incubated briefly in hyperosmolar solution were markedly enlarged. An otherwise identical experiment in which 150 mM mannitol was used instead of 150 mM sucrose similarly yielded caveolar enlargement (data not shown). Incubation for only 1 minute in the hypertonic medium containing 150 mM sucrose showed that a substantial fraction of myocyte caveolae were already enlarged. Incubation for 15 minutes in hypertonic solution containing 150 mM sucrose at the reduced temperature of 18°C likewise caused caveolar enlargement,

whereas at 18°C, the caveolae of the isotonic control atria were not enlarged (data not shown). Enlarged caveolar profiles were also present in atria incubated at 37°C in the hyperosmolar solution for the much longer duration of 44 minutes (Fig 8). The enlargement of atrial plasmalemmal caveolae in response to 150 mM sucrose was obvious, striking, and reproducible, and we found no examples where inspection of caveolae did not suffice to identify specimens thus treated.

Fig 9 shows electron micrographs of replicas of cross-fractured plasmalemmal caveolae from the two halves of the same atrium. One half atrium was exposed at 37°C for 5 minutes to 150 mM sucrose–containing
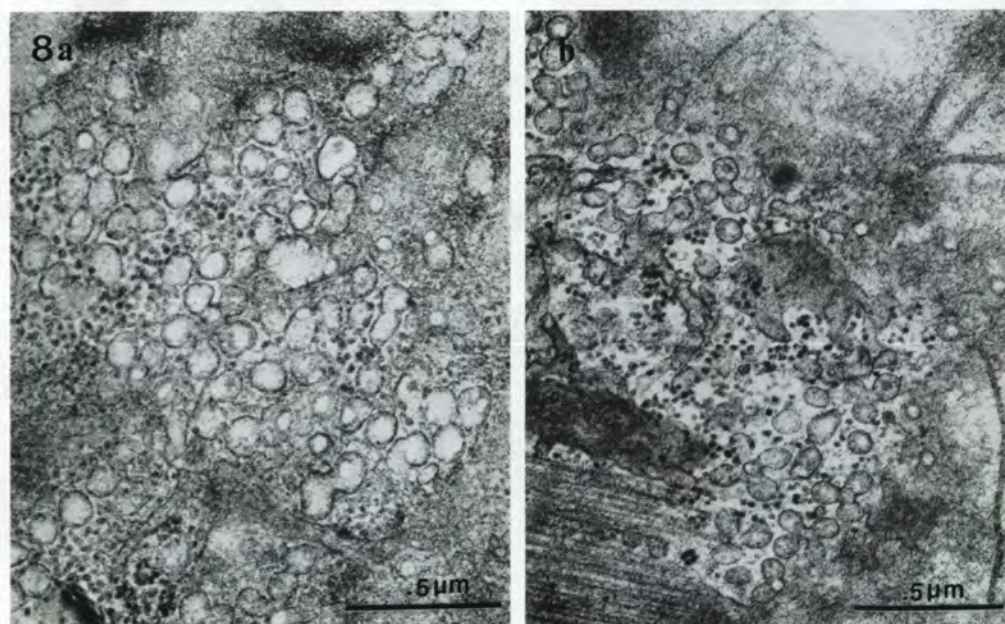

FIG 8.   *Electron micrographs of unstretched half atrium (panel a) and control half atrium (panel b). Experimental protocol was the same as for Fig 7, except that unstretched atrial halves were incubated for 44 minutes in hypertonic and control solutions. Note that enlargement of caveolar profiles in the hypertonic sucrose-treated atrial half (panel a) did not regress at long incubation time.*
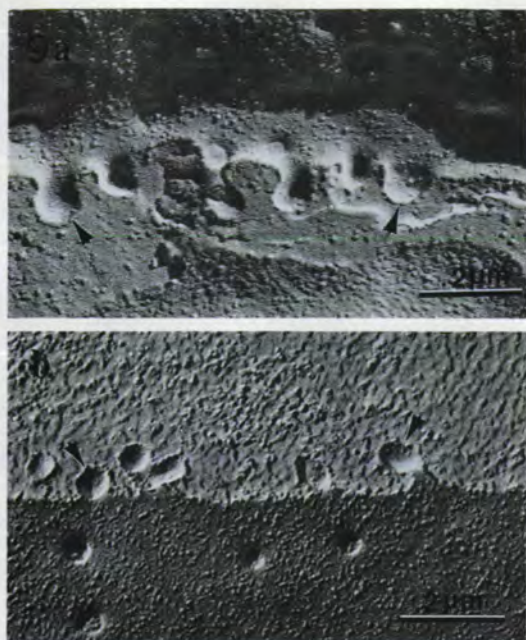
FIG 9. *Electron micrographs of replicas of freeze-fractured unstretched atrial halves prepared after 5 minutes in hypertonic medium containing 150 mM sucrose (panel a) and after 5 minutes in sucrose-free control medium (panel b). Note that cross-fractured caveolae (arrowheads) are larger in panel a than in panel b.*

hypertonic solution and fixed at the same osmolarity with glutaraldehyde. The other half of the same atrium (the isotonic control) was treated identically, except that 150 mM sucrose was omitted from the experimental and fixation solutions. Experiments like those shown in Fig 9 confirm that the increase in caveolar size can be observed by the freeze-fracture technique, which does not require exposure to OsO_4. Fig 10 (representative of results from two rats) shows electron micrographs of thin-sectioned preparations exposed for 5 minutes to 150 mM sucrose–containing hypertonic medium and then returned for 5 minutes to a solution with osmolar-

ity reduced to control levels (followed by fixation at control osmolarity). Fig 10b shows that caveolar enlargement had reversed within 5 minutes. One similar experiment (not illustrated) was used to show that caveolar enlargement was at least partially reversible within 1 minute.

Two additional experiments were performed on an atrial preparation stretched at 37°C for 5 minutes by a distending pressure of 5.1 mm Hg in the presence of the above-described 150 mM sucrose–containing hypertonic solution on both endocardial and epicardial surfaces. Electron micrographs of thin sections made after fixation at the same (hypertonic) osmolarity showed that the caveolae of atrial myocytes were enlarged, as in unstretched atria (data not shown). This result suggests that the reduced tension experienced by the plasma membrane of unstretched atria whose cell volume decreased by one third in 150 mM sucrose (hypertonic) medium (E. Page, J. Upshaw-Earley, and D.A. Hanck, unpublished observations) was not the factor responsible for caveolar enlargement, since enlargement was not prevented when the tension on the plasma membrane was increased by a hydrostatic distending pressure. Figs 11a and 11b show the cross-sectional diameters of the cross-fractured caveolar necks in freeze-fracture replicas of atrial halves treated with hypertonic solution and control solution, respectively. The figure suggests that exposure to the hypertonic sucrose-containing medium enlarged the average diameter of the fractured caveolar necks significantly, although the diameters of some necks exposed to hypertonic solutions were within the normal range (see next section).

Hyperosmolar Solution Increases Both the Number of Caveolar Necks Inserted per Unit Plasma Membrane Area and the Mean Cross-sectional Diameter of the Necks

Half atria from the same heart were exposed for 5 minutes either to control solution or to 150 mM sucrose–containing hyperosmolar solution. Electron micrographs of thin sections made from these differently treated atrial halves suggested that the hypertonic treatment might be increasing the number of caveolae. To
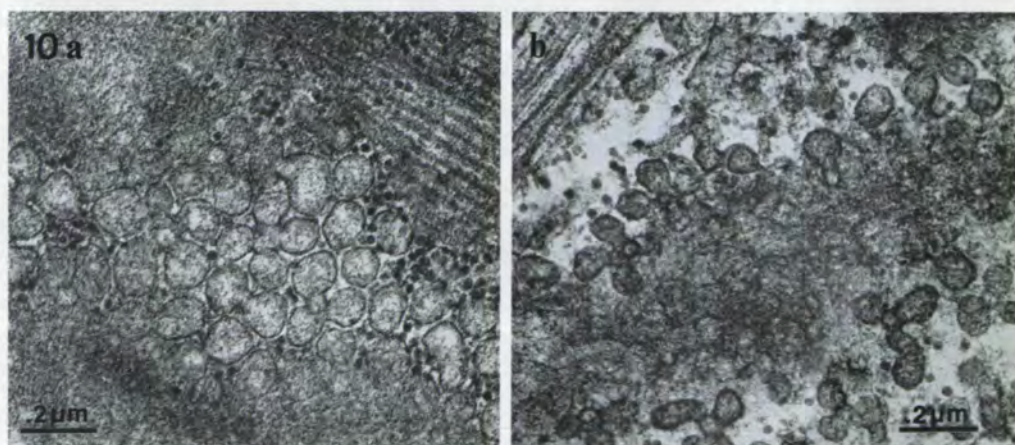

FIG 10. *Electron micrographs showing reversal of caveolar enlargement on return to control solution. Panel a: Characteristic caveolar swelling in myocyte of atrial half incubated 5 minutes at 37°C in hypertonic medium with 150 mM sucrose. Panel b: Normal-sized caveolar profiles of the other half atrium treated with hypertonic sucrose-containing solution, as described in panel a, and then incubated for 10 minutes at 37°C in control (sucrose-free) solution. Note normal-sized caveolar profiles.*
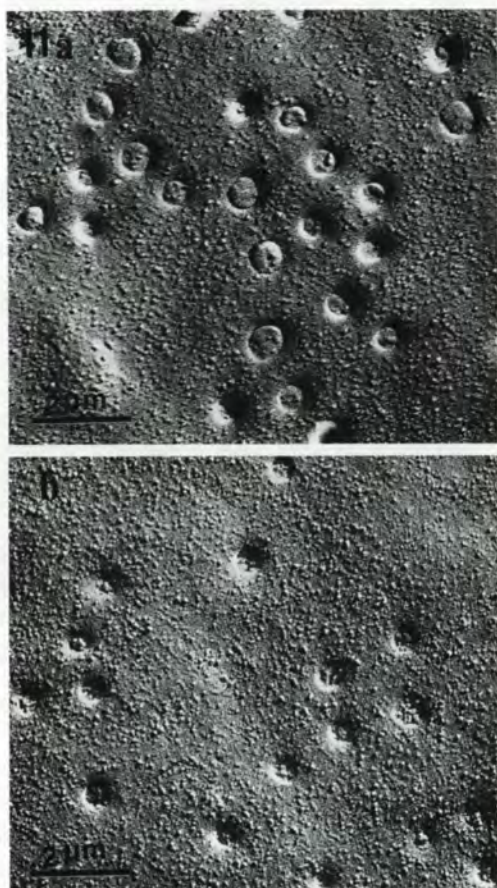
FIG 11.   *Electron micrographs of replicas of freeze-fractured unstretched atrial halves from the same atrium after 5 minutes in hypertonic medium containing 150 mM sucrose (panel a) and after 5 minutes in sucrose-free control medium (panel b). Note that fractured caveolar necks in panel a are larger and have a more distinct rim than in panel b even though both the direction of shadowing and the magnification are the same.*

quantify this impression by a more definitive method, we performed a systematic analysis (using computer-assisted image processing) of the surface density of fractured caveolar necks in replicas of freeze-fractured right atria previously cut into two pieces as described in "Materials and Methods." Fig 12 shows low-magnification survey electron micrographs of the plasmalemmal fracture faces of atrial halves in hypertonic and control solutions. Fig 12 and Fig 13, which displays graphically the results of quantitative analysis of caveolar neck surface densities in the plasmalemmal fracture faces of atrial myocytes subjected to image processing as described in "Materials and Methods," confirm that the surface density of caveolar necks (number of necks per square micrometer of fracture-face area) in the lipid bilayer of myocyte plasma membrane was indeed greater after a 5-minute exposure to the hyperosmolar medium. The experimental design, in which one of the two halves of the same atrium was incubated in control solution while the other half was incubated in the hyperosmolar solution of identical composition except for the addition of 150 mM sucrose, avoided variability between atria of different animals because each animal

(and each atrium) served as its own control. The precaution of carrying out the analysis on coded photographs with the observer blinded minimized observer bias. Fig 13 indicates that a brief (5-minute) exposure to the hyperosmolar solution did indeed increase the surface density of caveolae by a factor (mean±SEM) of 1.7 ± 0.1 and that the difference between the mean values of the hypertonic and control caveolar surface densities was significant ($P=.001$). The mean surface density decreased to control values 5 minutes after returning half atria, which had previously been exposed to 150 mM sucrose–containing hypertonic solution, to sucrose-free control solution (Fig 13). Fig 14 shows histograms of the diameters calculated from measured areas in control solution (left peak) and in the presence of sucrose (right peak). Of the 4500 observations, 3 were discarded as noise because they represented values below the resolution of the system. Because of the near equality in the sample populations and the experimental design that provided both control and sucrose data from each rat, we tested for the difference between sucrose and control groups with a paired t test. Mean values for caveolar diameter were calculated from the measurements for each atrium. These values and their standard deviations are shown on the Table. A paired t test established the likelihood that these mean diameters were different by chance alone at $P<.02$. It should be noted that in both control and sucrose data the distribution had a long tail on the side of larger diameters, which was more conspicuous in the sucrose data. This was expected because of the natural physical lower limit of caveolar neck dimension and because of the limit of resolution of the measuring technique. However, the tail did raise the possibility that the paired t test might give an incorrect evaluation of the probability that the populations were different because they were not distributed normally. To deal with this issue, we also log-transformed the data, which had the effect of normalizing the distributions, and then repeated the paired t test. The difference between the diameters was significant at $P<.01$ for the transformed data, confirming that the skew of the data did not spuriously affect the paired t test.

Since solutions made hypertonic by addition of 150 mM sucrose to control solutions decrease the volume of myocardial cells in mammalian heart muscle,[18,22] we performed two exploratory experiments on single atria to determine whether other agents that decrease the volume of atrial myocardial cells[22] would prevent the caveolae of myocardial cells from enlarging in response to 150 mM sucrose. Pretreatment for 10 minutes with 10 μM bumetanide failed to prevent enlargement of caveolae in response to 150 mM sucrose. Similarly, pretreatment for 10 minutes with 10 μM 8-bromoadenosine $3':5'$-cyclic monophosphate, which, like bumetanide, decreases myocardial cell volume,[22] also did not prevent caveolar enlargement in response to hypertonic medium containing 150 mM sucrose. Moreover, bumetanide and 8-bromoguanosine $3':5'$-cyclic monophosphate failed to produce clear-cut and reproducible caveolar size changes in the absence of 150 mM sucrose.

Preliminary experiments designed to produce caveolar enlargement by hypertonic 150 mM sucrose–containing solutions in 7- to 8-day-old primary cultures of atrial myocytes from the hearts of adult rats[21] were
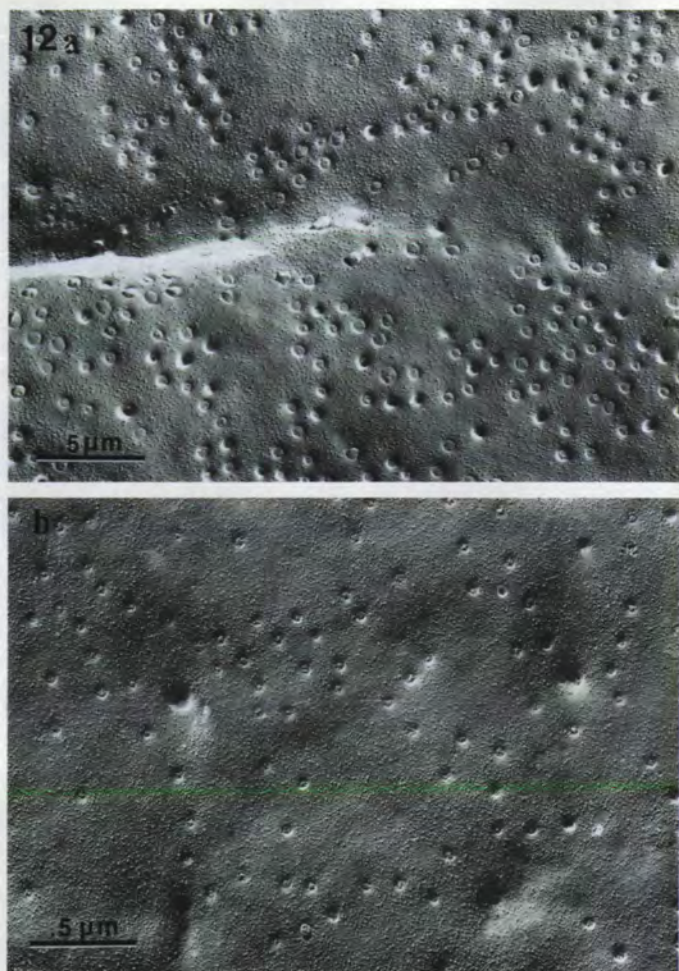
FIG 12. *Representative low-magnification survey electron micrographs of replicas of freeze-fractured unstretched atrial halves prepared under the same conditions described in Fig 11. Note increased surface density of caveolar necks (number of necks per area of fracture face) in the specimen exposed to hypertonic sucrose-containing medium (panel a) as compared with the specimen exposed to sucrose-free control medium (panel b).*

inconclusive. Although caveolae (both single and in the pinwheel configuration), as well as caveolar necks, were readily identifiable, caveolar enlargement (if any) was borderline and not consistently reproducible in electron micrographs of cultured myocytes fixed at defined osmolarity and then positively stained like the above-described intact atria.

Discussion
Ability of Caveolae in Atrial Myocytes to Open and Close

In the present study, we have examined the hypothesis that the caveolae of atrial myocytes can be either open to the interstitial space or sealed off from it, ie, that the open and closed states are alternative normal options associated with an as yet undetermined physiological role. "Closure" might consist of at least three options: (1) a reversible approximation of the "walls" of the caveolar necks or of the mouths of the caveolae where they join the caveolar necks, (2) a change in the permeability and/or position of the "diaphragms" so as to seal off the caveolar necks, or (3) withdrawal of the caveolar necks from the lipid bilayer by retracting them to the cytoplasmic surface of the caveolar membrane. As has been the experience of others who have studied caveolae of muscle cells (see review by Severs[6]), we have found no direct ultrastructural evidence to support the notion that the caveolae of atrial myocytes in situ can

exist in a closed state. However, we have demonstrated that both the number of caveolar necks inserted into the plasma membrane bilayer and the cross-sectional area of these necks can be varied experimentally. These observations suggest that caveolar necks can be both inserted into and withdrawn from the lipid bilayer and that both caveolae and their necks undergo reversible changes in volume.

Our inability and that of others to identify a closed state for caveolae of cardiac myocytes in electron micrographs does not, however, settle the argument. It is still a tenable hypothesis that, in vitro and in situ, caveolae normally cycle between the open and closed states at some frequency or, alternatively, that they cycle between states in which the caveolar necks are either inserted into or withdrawn from the lipid bilayer of the overlying myocyte plasma membrane. The approaches used to preserve caveolar structure for electron microscopy are chemical fixation, quick-freezing, or variations and combinations of these two methods.[6] It may be that these methods of preservation stabilize caveolar structure in the open state or in the neck-inserted state. That might happen for caveolae that cycle between open and closed states (1) if the duration of caveolar closure (or the neck-withdrawn state) were normally short relative to the duration of the open (or neck-inserted) state, (2) if the frequency of closure (or neck withdrawal) were normally low, (3) if the method of preservation shifted
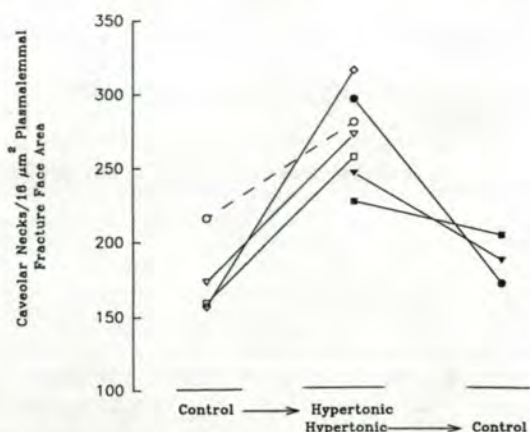
FIG 13.   *Graph showing the mean number of caveolar necks per unit area of the plasmalemmal fracture face. On the left is seen the effect of 5-minute exposure at 37°C to hypertonic (150 mM sucrose–containing) solution on surface density of caveolar necks in plasma membrane fracture faces of atrial myocytes from the two halves of the same rat right atrium (open symbols connected by continuous lines) or from right atria of different rats (open symbols connected by broken line). As explained in detail in the section on image processing in "Materials and Methods," the mean±SEM caveolar neck surface densities for 40 fracture-face areas in the control hemiatrium were calculated and compared with the mean±SEM caveolar neck surface densities for 40 fracture-face areas in the hypertonic sucrose-treated hemiatrium (from the same atrium and rat), for a total of three atria; similar calculations were made for right atrial halves from two different rats for one experiment only. For each of the four experiments, the mean values after hypertonic exposure were significantly larger than control values (P=.0001 to .001; area measured, 640 μm² [six half atria] or 480 μm² [two half atria]). On the right is seen the reversibility of increase in caveolar neck surface density after a 5-minute period of return to control solution (solid symbols), with mean±SEM calculated in the same way as above. Mean values were smaller after return to control solution in all three experiments, the reduction being highly significant (P<.003) in two of three experiments. The area measured was 640 μm² for each of six half atria. Mean±SEM for the transition from control to hypertonic (open symbols) were, respectively, as follows (in number of necks per 16 μm²): squares, from 160±10 to 259±11; diamonds, from 157±12 to 317±12; triangles, from 178±16 to 273±14; and circles, from 216±14 to 281±20. For the reversal experiments (transition from hypertonic to control [solid symbols]), the mean±SEM values were, respectively, as follows (in number of necks per 16 μm²): circles, from 297±22 to 172±12; triangles, from 247±14 to 188±14; and squares, from 228±9 to 205±14.*

caveolar configuration toward the open (or neck-inserted) state, or (4) for a combination of these reasons.

In this context, although the open and closed states of caveolae may correspond, respectively, to the neck-inserted and neck-withdrawn states, this relation need not necessarily hold. It seems reasonable to assume that, if a caveolar neck is not inserted into the plasma membrane, the interior of the caveolae to which that neck is connected cannot be in continuity with the interstitial space. However, without additional information, it cannot be assumed that all necks whose stumps appear in the lipid bilayer of the myocyte plasma membrane in replicas of freeze-fractured atria were necessarily open to the interstitial space. Indeed, one possible explanation of caveolar swelling in hypertonic sucrose-containing solutions requires the assumption that the hypertonic solutions close off the caveolae from the interstitial space (see following section).

It is important to design new methods to pursue these questions further in view of recent studies suggesting that caveolae in nonmuscle cells cycle between open and closed states as part of their physiological role in the binding of folate to a membrane receptor that is a glycosyl-phosphatidylinositol–anchored membrane protein.[10-13,23] In the model proposed by Rothberg et al[5] for this system, caveolae remain fixed in position beneath the plasma membrane (as caveolae of atrial myocytes are also thought to be[6]). However, Raposo et al[24,25] have presented evidence that β-adrenergic receptors and muscarinic acetylcholine receptors are internalized via apparently uncoated vesicles; Payne et al[26] found that the receptor for yeast mating factor uses uncoated vesicles for internalization; and Strosberg[27] has suggested that such internalization mechanisms may operate for G protein–linked receptors in general.

Possible Mechanisms for Caveolar Enlargement in Hypertonic Solutions

Exposure to hypertonic solutions (150 mM sucrose added to control solution) might bring about caveolar enlargement in at least three ways. One possible mechanism is that, if exposure to hypertonic solutions allows hypertonic sucrose to enter the caveolae and then somehow closes off the continuity between caveolar neck and interstitial space, the solution in the caveolae would become transiently hypertonic to the cytosol of the cardiac myocyte. The reason for this effect would be that the large volume of the cytosol would take much longer to come to osmotic equilibrium with the hyperosmotic interstitial compartment than the interior of the small superficially located caveolae. The caveolae would therefore undergo osmotic swelling as water diffuses from the cytosol across the caveolar membrane into the closed off caveolar lumen, where the activity (concentration) of water would be transiently lower than in the cytosol. The rapidity of caveolar enlargement and its reversibility within 1 minute are consistent with an osmotic mechanism, assuming that the caveolar membrane is rapidly water permeable. However, the interpretation, based on a combination of caveolar neck closure and osmotic swelling via water flow across the caveolar membrane, is inconsistent (without additional assumptions) with our observations that caveolae remain enlarged after 44 minutes in 150 mM hypertonic sucrose medium. Forty-four minutes should have sufficed to achieve osmotic equilibrium between the interstitial space, cytosol, and caveolar lumen. Caveolar swelling, if due to osmotic gradients, should therefore have regressed. It is conceivable that prolonged distension irreversibly damaged the mechanisms for restoring caveolar volume to control size. Without assuming such irreversibility, the proposed mechanism would not suffice to sustain an explanation based solely on osmotic flow of water across caveolar membranes.
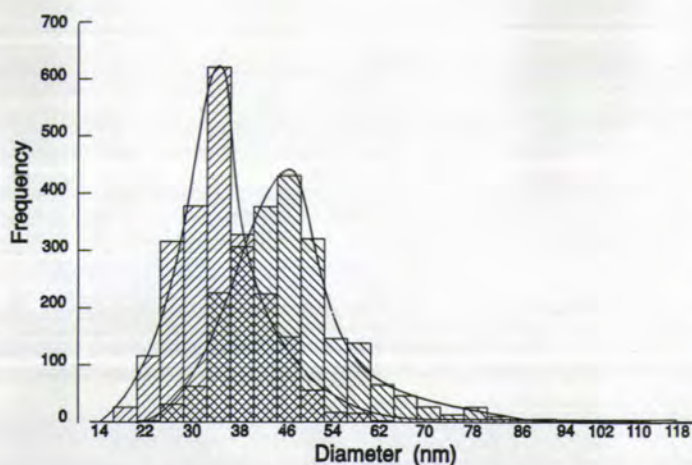
FIG 14. *Histogram showing the effect of a 5-minute exposure at 37°C to hypertonic (150 mM sucrose–containing) solution on the frequency distribution of caveolar neck cross-sectional diameters in the plasmalemmal fracture faces of the same replicas used to measure the caveolar neck surface densities graphed in Fig 13. See text for statistical treatment. Left peak, control; right peak, hypertonic sucrose. Lines were hand-drawn through the data for convenience of visualization.*

A second possible mechanism for caveolar enlargement might be the addition of caveolar membrane to existing caveolae by accretion, ie, by recruitment of membrane from an unidentified source of caveolar membrane precursor. Such a mechanism seems a priori less probable than osmotic swelling. Nevertheless, accretion might also be compatible with enlargement of caveolar necks. In heart and skeletal muscle, caveolar membrane is different from noncaveolar membrane in that membrane particles in the caveolar fracture face are absent or rare.[1,3] This finding reflects a paucity of large integral membrane proteins like assembled membrane channels or carriers. For enlargement by accretion of new membrane, the rapidity of the response to hypertonic solution implies that the membrane precursor should be available nearby. Conversely, the rapid reversibility of caveolar enlargement (within 1 minute) implies that membrane added to caveolae can be rapidly removed.

If the interiors of caveolae contain a matrix with ion-exchanger properties, sorption of sucrose (and associated water) is a third mechanism that might also contribute to caveolar swelling.[28]

Mechanism of the Increase in Surface Density of Caveolar Necks in Hypertonic Solutions

One plausible mechanism for the rapid increase in the surface densities of caveolar necks in atrial myocytes after exposure of the atria to solutions made hypertonic by adding 150 mM sucrose to control solution is an increase in the fraction of existing caveolae whose necks are inserted into and through the lipid bilayer of the overlying plasma membrane. This explanation presupposes that caveolae exist under the plasma membrane in at least two conformations—neck inserted and neck withdrawn and therefore uninserted. The idea that caveolar necks can be inserted into and withdrawn from

the plasma membrane suggests that cytoskeletal elements interacting with the cytoplasmic surface of the caveolar and/or caveolar neck membrane may be involved. Although speculative, this notion is consistent with the structures observed on the cytoplasmic surface of caveolae[29,30] and the isolation of the protein called caveolin.[10]

Another problem is why exposure to hypertonic sucrose should increase caveolar neck surface density and whether this phenomenon has counterparts in the normal functioning of caveolae. These questions are at present unanswerable, since the role of caveolae in atrial heart muscle cells is unknown. The cell water content shrinks by one third in a solution made hypertonic with 150 mM sucrose (E. Page, unpublished observation). Cell volume must therefore decrease commensurately. One possibility is that the increase in caveolar neck surface density may be a compensatory response stimulated by a decrease in cell volume of atrial myocytes, by the decreased activity of water in the extracellular solution that precedes cellular water loss, or by the decreased tension (stretch) of the plasma membrane as a consequence of the loss of cell water. To date, there is no direct evidence that the plasma membranes of cardiac myocytes contain highly selective water channels like those whose insertion into or withdrawal from epithelial cell plasma membranes is implicated in osmotic water permeability of epithelia,[31-33] but such water channels deserve to be systematically sought in both atrial and ventricular heart muscle cells. The near absence of intramembrane particles in the fracture faces of cardiac caveolae raises questions about whether caveolae may serve as targets for transient insertion of proteins involved in regulation of cell volume, movement of caveolar necks, or as yet unknown functions, perhaps involving inositol-1,4,5-trisphosphate receptors.[14] Recent studies suggest that recycling of atrial granule membranes is done by clathrin-coated vesicles,[4] not by caveolae. Thus, caveolae in atrial heart muscle cells may not be directly involved in endocytosis related to atrial natriuretic peptide secretion.

TABLE 1. Measurement of Calveolar Diameters

Rat No.	Mean calveolar diameter (nm)		Difference (nm)
	Control data	Sucrose data	
1	35.7±6.8	44.7±7.3	9.0
2	35.1±6.2	47.2±9.0	12.1
3	39.7±7.5	54.4±13.7	14.8

Values are mean±SEM.

References

1. Dulhunty AF, Franzini-Armstrong C. The relative contributions of the folds and caveolae to the surface membrane of frog skeletal muscle fibres at different sarcomere lengths. *J Physiol (Lond).* 1975;250:513-539.
2. Gabella G. Inpocketings of cell membrane (caveolae) in the rat myocardium. *J Ultrastruct Res.* 1978;65:135-147.
3. Levin KR, Page E. Quantitative studies on plasmalemmal folds and caveolae of rabbit ventricular myocardial cells. *Circ Res.* 1980; 46:244-255.
4. Newman TM, Severs NJ. Coated vesicles are implicated in the post-fusion retrieval of the membrane of rat atrial secretory granules. *Cell Tissue Res.* 1992;268:463-469.
5. Rothberg KG, Ying Y-S, Kamen BA, Anderson RGW. Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J Cell Biol.* 1990;111: 2931-2938.
6. Severs NJ. Caveolae: static inpocketings of the plasma membrane, dynamic vesicles or plain artifact? *J Cell Sci.* 1988;90:341-348.
7. Poulos AC, Rash JE, Elmund JK. Ultrarapid freezing reveals that skeletal muscle caveolae are semipermanent structures. *J Ultrastruct Mol Struct Res.* 1986;96:114-124.
8. Simionescu N. Cellular aspects of transcapillary exchange. *Physiol Rev.* 1983;63:1536-1560.
9. Page E, Upshaw-Earley J, Goings GE. Permeability of rat atrial endocardium, epicardium, and myocardium to large molecules: stretch-dependent effects. *Circ Res.* 1992;71:159-173.
10. Rothberg KG, Heuser JE, Donzell WC, Ying Y-S, Glenney JR, Anderson RGW. Caveolin, a protein component of caveolae membrane coats. *Cell.* 1982;68:673-682.
11. Glenney JR, Suppet D. Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus transformed fibroblasts. *Proc Natl Acad Sci U S A.* 1992;89:10517-10521.
12. Sztul E, Kaplin A, Saucan L, Palade G. Protein traffic between distinct plasma membrane domains: isolation and characterization of vesicular carriers involved in transcytosis. *Cell.* 1991;64:81-89.
13. Rothberg KG, Ying Y, Kolhouse JF, Kamen BA, Anderson RW. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. *J Cell Biol.* 1990;110:637-649.
14. Fujimoto T, Nakade S, Miyawaki A, Mikoshiba A, Ogawa K. Localization of inositol 1,4,5-triphosphate receptor-like protein in plasmalemmal caveolae. *J Cell Biol.* 1992;119:1507-1513.
15. Page E, Goings GE, Power B, Upshaw-Earley J. Basal and stretch-augmented natriuretic peptide secretion by quiescent rat atria. *Am J Physiol.* 1990;259(*Cell Physiol* 28):C801-C818.
16. Page E, Upshaw-Earley J, Goings GE, Hanck DA. Effect of external Ca^{2+} concentration on stretch-augmented natriuretic peptide secretion by rat atria. *Am J Physiol.* 1991;260(*Cell Physiol* 29):C756-C762.
17. Page E, Upshaw-Earley J, Goings GE, Hanck DA. Inhibition of atrial peptide secretion at different stages of the secretory process. *Am J Physiol.* 1991;261(*Cell Physiol* 30):C1162-C1176.
18. Page E, Storm SR. Cat heart muscle in vitro, IX: cell ion and water contents in anisosmolal solution. *J Gen Physiol.* 1966;49:641-653.
19. Page E, Goings GE, Power B, Upshaw-Earley J. Ultrastructural features of atrial peptide secretion. *Am J Physiol.* 1986;251(*Heart Circ Physiol* 20):H340-H348.
20. Kordylewski L, Karrison T, Page E. Measurements on the internal structure of freeze-fractured cardiac plasma membrane. *Am J Physiol.* 1985;248(*Heart Circ Physiol* 17):H297-H304.
21. Iida H, Shibata Y. Delivery of lectin-labeled membrane to the trans-Golgi network and secretory granules in cultured atrial myocytes. *J Histochem Cytochem.* 1989;37:1885-1892.
22. Clemo HF, Feher JJ, Baumgarten CM. Modulation of rabbit ventricular cell volume and $Na^+/K^+/2Cl^-$ cotransport by cGMP and atrial natriuretic factor. *J Gen Physiol.* 1992;100:89-114.
23. Kamen BA, Wang M-T, Streckfuss AJ, Peryea X, Anderson RGW. Delivery of folates to the cytoplasm of MA 104 cells is mediated by a surface membrane receptor that recycles. *J Biol Chem.* 1988;263: 13602-13609.
24. Raposo G, Dunia I, Delavier-Klutchko C, Kaveri S, Strosberg AD, Benedeth EL. Internalization of β-adrenergic receptor in A431 cells involves non-coated vesicles. *Eur J Cell Biol.* 1989;50:340-352.
25. Raposo G, Dunia I, Marvelo S, Andre C, Guillet JG, Strosberg AD, Benedeth EL, Hoebeke J. Redistribution of muscarinic acetylcholine receptors on human fibroblasts induced by regulatory ligands. *Biol Cell.* 1987;60:117-124.
26. Payne GS, Baker D, Van Tuinen E, Scheckman R. Protein transport to the vacuole and receptor-mediated endocytosis by clathrin-deficient yeast. *J Cell Biol.* 1988;106:1453-1456.
27. Strosberg AD. Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. *Eur J Biochem.* 1991;196:1-10.
28. Helfferich F. *Ion Exchange.* New York, NY: McGraw-Hill Book Co; 1962:131.
29. Izumi T, Shibata Y, Yamamoto T. The cytoplasmic surface structures of uncoated vesicles in various tissues of rat as revealed by quick-freeze, deep-etching replicas. *J Electron Microsc (Tokyo).* 1989;38:47-53.
30. Peters KR, Carley WW, Palade GE. Endothelial plasmalemmal vesicles have a characteristic striped bipolar surface structure. *J Cell Biol.* 1985;101:2233-2238.
31. Harris HW, Handler JS. The role of membrane turnover in the water permeability response to antidiuretic hormone. *J Membr Biol.* 1988;103:207-216.
32. Harris HW, Zerkel ML, Hosselet C. Quantitation and topography of membrane proteins in highly water-permeable vesicles from ADH-stimulated toad bladder. *Am J Physiol.* 1991;261:C143-C153.
33. Verkman AS. Mechanisms and regulations of water permeability in renal epithelia. *Am J Physiol.* 1989;257:C837-C850.