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β-Subunit of cardiac Na^+-K^+-ATPase dictates the concentration of the functional enzyme in caveolae

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Liu, Lijun, and Amir Askari. β-Subunit of cardiac Na^+-K^+-ATPase dictates the concentration of the functional enzyme in caveolae. *Am J Physiol Cell Physiol* 291: C569–C578, 2006. First published April 19, 2006; doi:10.1152/ajpcell.00002.2006.—Previous studies showed the presence of a significant fraction of Na^+-K^+-ATPase α-subunits in cardiac myocyte caveolae, suggesting the caveolar interactions of Na^+-K^+-ATPase with its signaling partners. Because both α- and β-subunits are required for ATPase activity, to clarify the status of the pumping function of caveolar Na^+-K^+-ATPase, we have examined the relative distribution of two major subunit isoforms (α_1 and β_1) in caveolar and noncaveolar membranes of adult rat cardiac myocytes. When cell lysates treated with high salt (Na_2CO_3 or KCl) concentrations were fractionated by a standard density gradient procedure, the resulting light caveolar membranes contained 30–40% of α_1-subunits and 80–90% of β_1-subunits. Use of Na_2CO_3 was shown to inactivate Na^+-K^+-ATPase; however, caveolar membranes obtained by the KCl procedure were not denatured and contained ~75% of total myocyte Na^+-K^+-ATPase activity. Sealed isolated caveolae exhibited active Na^+ transport. Confocal microscopy supported the presence of α,β-subunits in caveolae, and immunoprecipitation showed the association of the subunits with caveolin oligomers. The findings indicate that cardiac caveolar inpocketings are the primary portals for active Na^+-K^+ fluxes, and the sites where the pumping and signaling functions of Na^+-K^+-ATPase are integrated. Preferential concentration of β_1-subunit in caveolae was cell specific; it was also noted in neonatal cardiac myocytes but not in fibroblasts and A7r5 cells. Uneven distributions of α_1 and β_1 in early and late endosomes of myocytes suggested different internalization routes of two subunits as a source of selective localization of active Na^+-K^+-ATPase in cardiac caveolae.

cardiac myocyte; caveolin; oligomer; ouabain; sodium pump

Na^+-K^+-ATPase is an oligomer of intrinsic membrane proteins that catalyzes the coupled active transport of Na^+ and K^+ and maintains the normal physiological gradients of these ions across the plasma membrane of most animal cells (16, 37). Two subunits of the enzyme (α and β) are essential for ATPase and ion pumping function, and some preparations also contain a member of the FXYD group of proteins that regulate function (6, 16, 39). In recent years, it has been realized that, in addition to pumping ions, Na^+-K^+-ATPase also acts as a signal transducer, i.e., the enzyme interacts with neighboring membrane proteins, such as Src and EGFR, to relay messages to intracellular signaling complexes and the nucleus (44). Our recent studies on the signal transducing function of Na^+-K^+-ATPase in cardiac myocytes (23) showed the presence of a significant fraction of the enzyme's α-subunits in the caveolae microdomains of the plasma membrane and suggested that the caveolar pool of the enzyme may be responsible for its signaling function because of its proximity to the signaling partners within the restricted space of the caveolae microdomain. This raised the question of whether or not the caveolar Na^+-K^+-ATPase also has ATPase activity and ion pumping functions. The present studies were initiated to address this question. Unexpectedly, our findings show that, because of the almost exclusive localization of the β-subunit of the cardiac enzyme in caveolae, the catalytic/pumping function of the enzyme is largely restricted to these sarcolemmal microdomains of the cardiac myocytes.

MATERIALS AND METHODS

Materials. Chemicals of the highest purity and culture media were purchased from Sigma (St. Louis, MO). Antibodies against the indicated antigens were obtained from the following vendors: α_1-, α_2-, β_1-, and β_3-subunits of Na^+-K^+-ATPase, EEA1, and Src (clone GD11) from Upstate Biotechnology (Lake Placid, NY); Rab5A (S-19), Rab7 (H50), sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) 2 (N-19), extracellular signal-regulated kinase (ERK) 1/2, goat anti-rabbit IgG-horseradish peroxidase (HRP), and goat anti-mouse IgG-HRP from Santa Cruz Biotechnology (Santa Cruz, CA); caveolin-1 (clone C060), caveolin-2 (clone 65), caveolin-3 (clone 26), and clathrin heavy chain from BD Transduction Laboratories (Lexington, KY); Na^+-K^+-ATPase α_1 (α6F) from Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA); vimentin, sarcomeric tropomyosin, and smooth muscle heavy chain from Sigma; and von Willebrand factor from DAKOPATTS (Copenhagen, Denmark). Customer-designed antibody against residues 58–72 of phospholemman (26) was produced by Sigma-Genosys (Woodlands, TX). Protein A/G plus agarose was purchased from Santa Cruz Biotechnology. $^{22}Na^+$ was purchased from Perkin-Elmer Life Sciences (Boston, MA). Kidney medullas were prepared by dissection from kidneys. Pig kidneys were obtained from a slaughterhouse. All research on rats was done according to procedures and guidelines approved by the Institutional Animal Care and Use Committee.

Cell preparation and culture. Calcium-tolerant adult rat cardiac myocytes were prepared from isolated hearts as described before (45). Freshly made suspensions were used for fractionation and caveolae isolation. Cardiac fibroblasts were obtained from the same hearts used for myocyte preparation. After collagenase treatment of the hearts, the disaggregated myocytes and nonmyocytes were separated by gravity (4), and fibroblasts were further purified and cultured as described previously (5). Only *passage 2–3* cells that were positive for vimentin but negative for von Willebrand factor, smooth muscle myosin heavy chain, and sarcomeric tropomyosin were used. Neonatal rat cardiac myocytes were prepared from ventricles of 1- to 2-day-old rats and

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cultured as previously described (30). A7r5 cells (American Type Culture Collection) were cultured as noted before (22).

Fractionation of cell lysates for the preparation of caveolar and noncaveolar membranes. The carbonate-based and detergent-free procedure of Song et al. (38) was used as described before (23). Briefly, cells were lysed at 4°C in a solution containing 0.5 M Na_2CO_3 (pH 11), 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 10 nM okadaic acid, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The samples were then homogenized, sonicated, and centrifuged by flotation through sucrose gradients at 4°C as indicated (23). After collection of twelve 1-ml fractions, each fraction was diluted in 10 vol of MBS [25 mM 2-(*N*-morpholino)ethanesulfonic acid, 150 mM NaCl, pH 6.5] and centrifuged at 100,000 *g* for 1 h at 4°C. The membrane pellet from each fraction was resuspended in MBS and used or frozen. A modification of the above procedure designed to avoid the denaturing effect of carbonate (see RESULTS) involved the replacement of 0.5 M Na_2CO_3 with 0.5 M KCl, prepared in MBS, in both the lysis and the gradient solutions. When ATPase activity was to be assayed in the fractionated membranes, Na_3VO_4 and NaF were omitted from the lysis solution.

Preparation of endosomes. Membrane fractions enriched in early and late endosomes were prepared from neonatal rat cardiac myocytes by the procedures of Gorvel et al. (11) as described by Liu et al. (20).

Other membrane preparations. Microsomes from rat and pig kidney medullas were prepared by previously described procedures (31). A crude membrane preparation was obtained from adult cardiac myocytes by suspending Ca^{2+}-tolerant myocytes isolated from rat hearts in 15 ml of a solution containing 0.25 M sucrose, 30 mM histidine, and 1 mM EDTA. The suspension was homogenized by a Teflon-glass homogenizer, further disrupted by a Polytron homogenizer, filtered through gauze, and centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was then centrifuged for 1 h at 115,000 *g* at 4°C. The sediment was suspended in the above sucrose solution and used or stored frozen. To obtain a crude adult rat heart membrane preparation, one ventricle was minced and then processed as described above.

Immunofluorescence microscopy. Immunofluorescent staining of myocytes attached to coverslips was conducted as described before (22, 23) with minor modifications. Briefly, cells were fixed with 2% paraformaldehyde for 10 min at room temperature and then permeabilized in 0.0025% saponin for 10 min. Cells were blocked by Image-iT FX signal enhancer (Molecular Probes) for 30 min. Incubations involving both monoclonal and polyclonal primary antibodies were done simultaneously overnight at 4°C. The following pairs of antibodies were used: anti-Na^+-K^+-ATPase β_1-monoclonal antibody (1:50; Upstate Biotechnology) and anti-caveolin-3 polyclonal antibody (1:200; Affinity BioReagents); anti-Na^+-K^+-ATPase α_1-monoclonal antibody (1:50; Upstate Biotechnology) and anti-Na^+-K^+-ATPase β_1-polyclonal antibody (1:100: Upstate Biotechnology). Antigen-antibody complexes were visualized with Alexa Fluor 488- or 546-conjugated secondary antibodies. The cover slips were mounted with ProLong Gold antifade reagent (Molecular Probes). Confocal images were detected by a Leica TCS SP2 spectral confocal scanner and a Leica DMIRE2 microscope (Leica, Mannheim, Germany) equipped with a ×63 oil-immersion objective. To avoid cross talk between the two fluorescence dyes, we used the sequential method in which the two laser lines 488 and 546 nm were applied to the cells alternatively. Visualization and analysis were performed using Leica Confocal Microscope Systems software.

Immunoblot analysis and immunoprecipitation. Membrane samples were subjected to SDS-PAGE and probed with appropriate antibodies as described before (23). For quantitative comparisons, different dilutions of the samples were used to ensure that quantitations were made within the linear range of the assays (e.g., see Fig. 2*A*).

For immunoprecipitation experiments, isolated adult cardiac myocytes, cultured neonatal myocytes, or caveolar membranes prepared from myocytes were treated with ice-cold lysis buffer containing 150 mM NaCl, 10 mM Tris·HCl (pH 8.0), 1% Triton X-100, 60 mM octylglucoside, 1 mM PMSF, 1 mM Na_3VO_3, 1 mM NaF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After 30 min at 4°C, the lysate was clarified by centrifugation at 16,000 *g* for 15 min. The supernatant (0.25–1 mg protein) was precleared and incubated with the appropriate antibody and then with protein A/G plus agarose beads. The proteins bound to the collected beads were subjected to SDS-PAGE and probed with appropriate antibodies as indicated above.

Na^+-K^+-ATPase activity. The various membrane preparations were assayed at 37°C by measuring the initial rate of release of P_i from ATP in a medium containing (in mM) 100 NaCl, 25 KCl, 3 $MgCl_2$, 1 EGTA, 2 ATP, and 20 Tris·HCl (pH 7.4). Each assay was done in the presence and absence of 1 mM ouabain, and the ouabain-sensitive component was considered as Na^+-K^+-ATPase activity. When indicated, alamethacin was included in the assay as described before (46). P_i was measured with Malachite Green (17), using the commercially available Biomol Green reagent (BIOMOL Research Laboratories, Plymouth Meeting, PA).

Na^+ transport assay. ATP-dependent Na^+ uptake by inside-out membrane vesicles was measured by modification of a procedure described before (47). Caveolar membranes of adult cardiac myocytes prepared by the KCl procedure (see above) were suspended in a solution containing 160 mM choline chloride, 2 mM KCl, and 20 mM MOPS (pH 7.2) at a protein concentration of 2–4 mg/ml. A sample (20 μl) was added to 80 μl of a solution containing (in mM) 150 choline chloride, 5 $^{22}NaCl$, 0.2 EGTA, 3 $MgCl_2$, 2 ATP, and 20 MOPS (pH 7.2). ATP was omitted from the control to measure ATP-independent uptake. After incubation at 24°C for 15 min, 3 ml of ice-cold stop solution containing 160 mM KCl and 20 mM MOPS (pH 7.2) was added, and the mixture was passed through a 0.45-μm Millipore filter. The filter was washed three times with the stop solution and counted. Membranes pretreated with 0.1 mM Na_3VO_3 or 0.1 mM digitoxigenin (47) were used to test the sensitivity of the ATP-dependent component of the Na^+ transport to these established inhibitors of Na^+-K^+-ATPase (37).

Other assays. Cholesterol contents of various membrane fractions were assayed by the fluorescent Amplex red cholesterol assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions, using a plate reader. Protein was measured using DC colorimetric assay (Bio-Rad, Hercules, CA).

Analysis of data. Unless stated otherwise, data are means ± SE of the results of a minimum of three experiments. Student's *t*-test was used, and significance was accepted at $P < 0.05$.

RESULTS

Na^+-K^+-ATPase subunits in caveolar and noncaveolar membranes of adult cardiac myocytes. We showed before, using rat cardiac ventricular muscle and isolated cardiac myocytes, that, when detergent-free fractionation procedures are used to prepare caveolar membranes, ~30% of the cellular contents of the α-subunit isoforms of Na^+-K^+-ATPase are retained in these plasma membrane microdomains (23). In our previous studies, although we also demonstrated the presence of the β-subunit of the enzyme in caveolae (23), we did not compare the relative distributions of the two subunits in caveolar and noncaveolar membranes. To do this, we extended our previous work, focusing on isolated adult rat cardiac myocytes.

The most widely used detergent-free procedures for the biochemical isolation of cardiac caveolar membranes are variations of the carbonate-based procedure of Song et al. (38). We used this procedure to fractionate the whole cell lysates of isolated adult cardiac myocytes. However, because our primary

interest was on membrane proteins, we sedimented the membrane component of each fraction, assayed the membrane fractions for protein and cholesterol, and used specific antibodies to detect the distribution of the Na^+-K^+-ATPase subunits and caveolins-2 and -3 in these membrane fractions (Fig. 1). The low buoyant density membranes (fractions 4 and 5) that were rich in cholesterol and contained most of the cellular content of caveolin-3 and -2 also contained significant quantities of the α_1- and α_2-subunits of Na^+-K^+-ATPase (Fig. 1), in agreement with our prior findings (23). Surprisingly, however, the β_1-subunit seemed to be quite restricted to caveolar membranes (Fig. 1). That the immunoreactive band designated as β_1 is indeed this subunit was confirmed as indicated before (23) by its complete conversion to a band of higher mobility (~35 kDa) upon deglycosylation (data not shown). In agreement with previous observations of others (25), we did not detect the β_2-isoform of Na^+-K^+-ATPase in rat cardiac myocytes. The β_3-isoform was detected in these myocytes as expected from the previous report of the presence of small quantities of β_3 in the rat heart (1); however, this minor isoform was also restricted to caveolae (Fig. 1). In the adult rat cardiac myocytes, α_1 and α_2 are the only isoforms present, with the former constituting ~80% of the total α-subunit (24, 45).

The immunoblots of Fig. 1 also show the distribution of some signaling partners of Na^+-K^+-ATPase (Src and ERK1/2) and a number of marker proteins in the membranes fractionated by the carbonate-based procedure. The results indicate that the noncaveolar membranes (fractions 6–12) obtained by this procedure consist of mixtures of sarcoplasmic reticulum (SERCA2), endosomes (Rab5, Rab7), and noncaveolar plasma membrane (clathrin). Certainly, they must also contain other internal membranes for which markers were not used, e.g., the mitochondria. Of interest is the distribution pattern of the sarcolemmal protein phospholemman (FXYD1), which is known to regulate the cardiac Na^+-K^+-ATPase (39); its distribution seems to be more similar to that of the α-subunit.

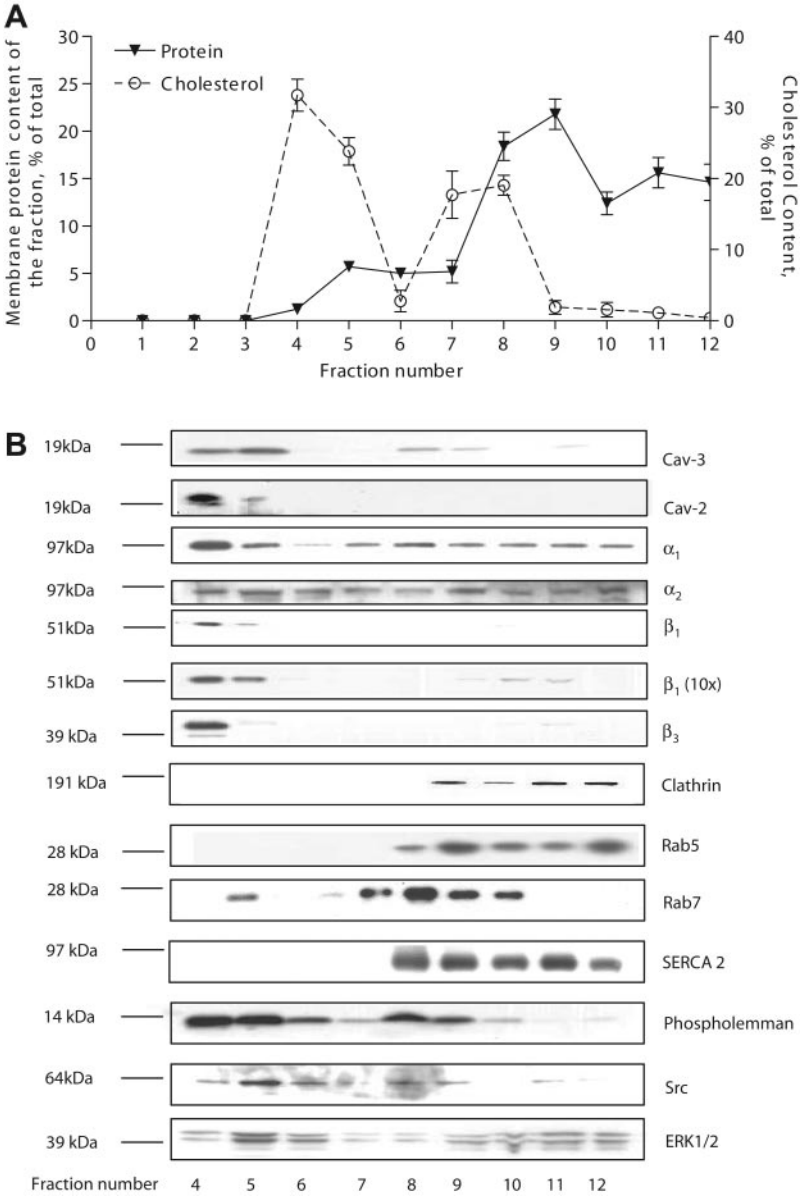

Fig. 1. Localization of the subunits of Na^+-K^+-ATPase and some marker proteins and signaling partners in caveolar and noncaveolar membranes of adult rat cardiac myocytes. Whole cell lysates of isolated myocytes were fractionated by the carbonate-based procedure, and membranes were obtained from each of the 12 collected fractions, assayed for protein and cholesterol, and subjected to Western blot analysis with specific antibodies. *A*: protein/cholesterol of 5 fractionated myocyte preparations (means ± SE). *B*: typical blots of one fractionated preparation. The membrane pellet from each fraction was suspended in the same volume of buffer, and an equal volume of each suspension was used for Western blots. Cav, caveolin; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; ERK, extracellular signal-regulated kinase.

To quantitate the relative distributions of the muscle-specific caveolin-3 and the two major subunits of Na^+-K^+-ATPase (α_1 and β_1) in caveolar and noncaveolar membranes, blots such as those shown in Fig. 1 were obtained from five fractionated myocyte samples and quantitated as described in MATERIALS AND METHODS. The results (Fig. 2) show the uneven distribution of the ATPase subunits in caveolar and noncaveolar pools. Interestingly, these data suggest that, in myocytes, the β_1-subunit may be a better marker of the caveolar membrane than caveolin-3. That in adult rat cardiac myocytes a significant fraction of caveolin-3 is in heavy noncaveolar membranes has also been noted recently by others (12).

In our previous studies on adult cardiac myocytes (23), we used immunostaining and confocal microscopy to provide support for the partial colocalization of the α_1- and α_2-subunits of Na^+-K^+-ATPase with caveolin-3 at peripheral sarcolemma and T tubules. In similar experiments (Fig. 3), we explored the subcellular locations of the β_1-subunits and their proximities to caveolin-3 and the α_1-subunits. The sample images clearly indicate colocalization of the β_1 with caveolin-3 and α_1, particularly in T tubules (Fig. 3). Considering the well-established existence of α,β association, these findings are consistent with the fractionation data (Figs. 1–2), indicating the presence of α,β-subunits in some, if not all, of the cardiac caveolae. That in adult rat cardiac myocytes there are distinct populations of caveolae containing different occupants has been suggested (36).

Inactivation of Na^+-K^+-ATPase by Na_2CO_3. In view of the preferential localization of the β-subunit in the caveolar membranes, and because the β-subunit is required for catalytic function, we wished to assay Na^+-K^+-ATPase activities of the different membrane pools obtained by the carbonate-based fractionation procedure. Cell lysates that are subjected to this fractionation procedure often contain vanadate and fluoride to inhibit tyrosine phosphatases. These agents, however, are also high-affinity or irreversible inhibitors of Na^+-K^+-ATPase (34, 37). When these inhibitors were left out of the lysis buffer (MATERIALS AND METHODS), the relative distributions of Na^+-K^+-ATPase subunits were the same as those presented in Fig. 1; however, we still could not detect significant ouabain-sensitive Na^+-K^+-ATPase activity in any of the fractions. This suggested that the use of Na_2CO_3 (pH 11) in the fractionation procedure may cause inactivation of the enzyme. To test this directly, we compared Na^+-K^+-ATPase activities of a crude rat heart membrane preparation and kidney microsomes (preparations with vastly different specific activities) before and after exposure to Na_2CO_3. The results (Fig. 4) showed that such exposure indeed causes significant inactivation of the enzyme. We were prompted, therefore, to modify the carbonate-based fractionation procedure to avoid the denaturation of Na^+-K^+-ATPase.

Na^+-K^+-ATPase subunits and activities in caveolar and noncaveolar membranes obtained by a nondenaturing procedure. Sodium carbonate was used for the development of a detergent-free procedure for the preparation of caveolar membranes (38) because of the established use of this salt to remove loosely attached peripheral membrane proteins from membranes containing intrinsic membrane proteins. Because of the well-known similar properties of high concentrations of other salts, and previous experience with the use of high-KCl concentrations for the partial purification of cardiac Na^+-K^+-ATPase (e.g., see Ref. 14), we modified the carbonate-based

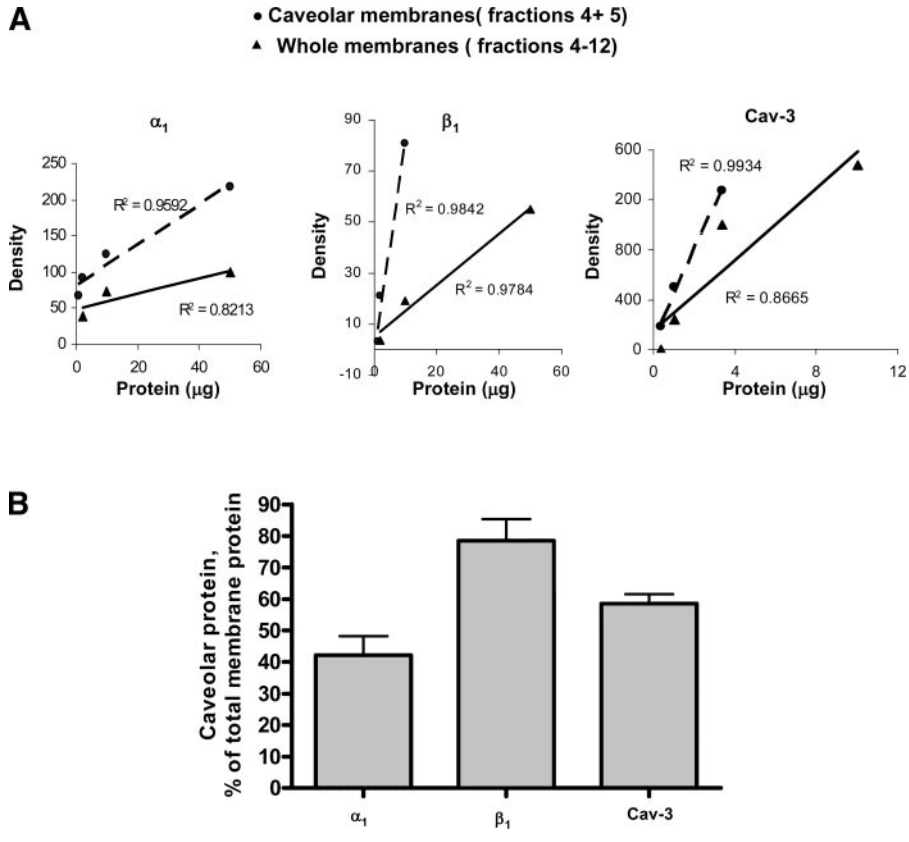

Fig. 2. Relative distribution of α_1- and β_1-subunits of Na^+-K^+-ATPase and caveolin-3 in caveolar and noncaveolar membranes of adult cardiac myocytes. Fractionated membranes were obtained as indicated in Fig. 1. *A*: caveolar membranes (*fractions 4* and *5*) and whole membranes (combined *fractions 4–12*) of a fractionated myocyte preparation were assayed for total protein, and different dilutions were immunoassayed for the indicated proteins to obtain the caveolar/whole membrane ratio of each protein within the linear range of the assay. R^2, goodness of fit. *B*: caveolar content of the indicated proteins based on caveolar/whole membrane ratio in 5 fractionated myocyte preparations.

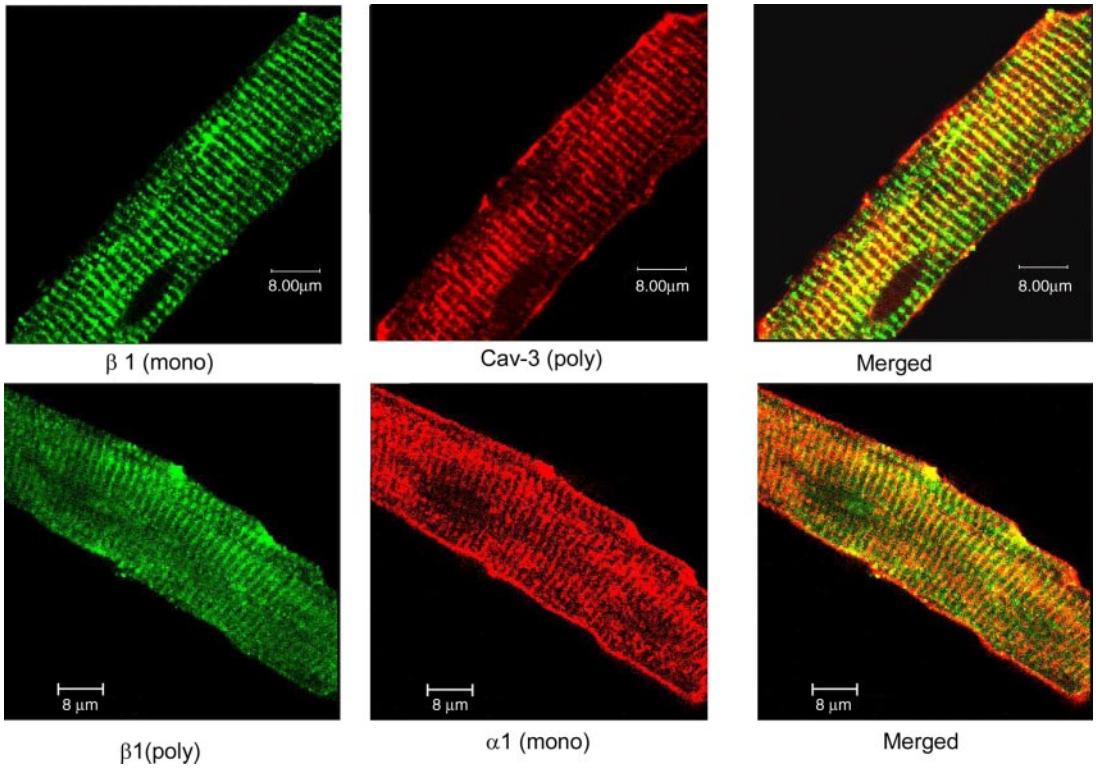

Fig. 3. Colocalization of Na^+-K^+-ATPase β_1-subunit with caveolin-3 (*top*) and α_1-subunit (*bottom*). Experiments were done as indicated in MATERIALS AND METHODS.

procedure by substituting 0.5 M KCl for 0.5 M Na_2CO_3 and assayed the resulting fractions for total protein, cholesterol, and immunoreactive proteins. The relative distributions of membrane protein, membrane cholesterol, caveolin-3, α_1-subunit, and β_1-subunit (Fig. 5) were similar to those obtained in the carbonate-based procedure (Figs. 1 and 2). The distributions of several marker proteins in the KCl fractions (not shown) were also the same as those shown in Fig. 1.

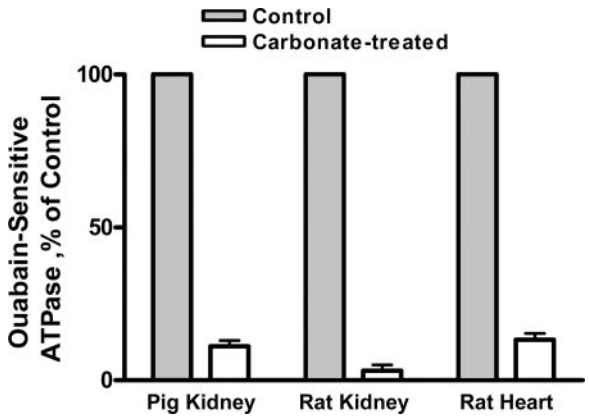

Fig. 4. Inactivation of Na^+-K^+-ATPase by sodium carbonate. Crude microsomes from pig kidney and rat kidney and a crude membrane preparation from rat heart were prepared as indicated in MATERIALS AND METHODS. Membranes suspended in *buffer A* (0.25 M sucrose, 30 mM histidine, and 1 mM EDTA, pH 7.4) were mixed with an equal volume of 1 M Na_2CO_3 or *buffer A* (control), sonicated on ice, diluted 10 times in 25 mM MES, 150 mM NaCl, pH 6.5, and centrifuged at 100,000 *g* for 1 h at 4°C. The sediments were suspended in *buffer A* and assayed as described in MATERIALS AND METHODS. The specific activities (in $\mu mol \cdot mg^{-1} \cdot h^{-1}$) of the controls were (means ± SE): pig kidney, 32.67 ± 4.7 ($n = 3$); rat kidney, 9.48 ± 0.4 ($n = 4$); rat heart, 2.59 ± 0.24 ($n = 4$).

In experiments, the results of which are summarized in Fig. 6, the caveolar and noncaveolar membrane fractions obtained by the KCl procedure were assayed for ouabain-sensitive Na^+-K^+-ATPase activity in the absence and in the presence of alamethacin to check for the presence of sealed vesicles (46). Both fractions exhibited activity, but the specific activity of the caveolar fraction was far greater than that of the noncaveolar fraction, and only the activity of the caveolar fraction was increased significantly by alamethacin. Based on these data, and the data of Fig. 5 on the membrane protein content of the various fractions, it may be estimated that >75% of the total Na^+-K^+-ATPase activity of adult myocytes resides in caveolae.

The significant increase in Na^+-K^+-ATPase activity of the caveolar membranes caused by alamethacin (Fig. 6) suggested that a fraction of these isolated membranes is sealed. This was confirmed by the data of Fig. 7 showing the presence of an ATP-dependent $^{22}Na^+$ uptake in these membranes that is sensitive to vanadate or digitoxigenin, two known inhibitors of Na^+-K^+-ATPase (37). Evidently, some caveolae are either sealed in the intact cell or form inside-out sealed vesicles in the course of the isolation procedure. That in the rat cardiac myocytes caveolae may alternate between open and closed off states has been suggested by Page et al. (28).

The Molar Ratio of α/β. Although the data of Figs. 1 and 2 clearly show that the ratio of α/β is lower in caveolar than in noncaveolar membranes, they do not provide information on the molar ratio of the two subunits in either pool. To address this issue, we took advantage of the fact that the molar ratio of

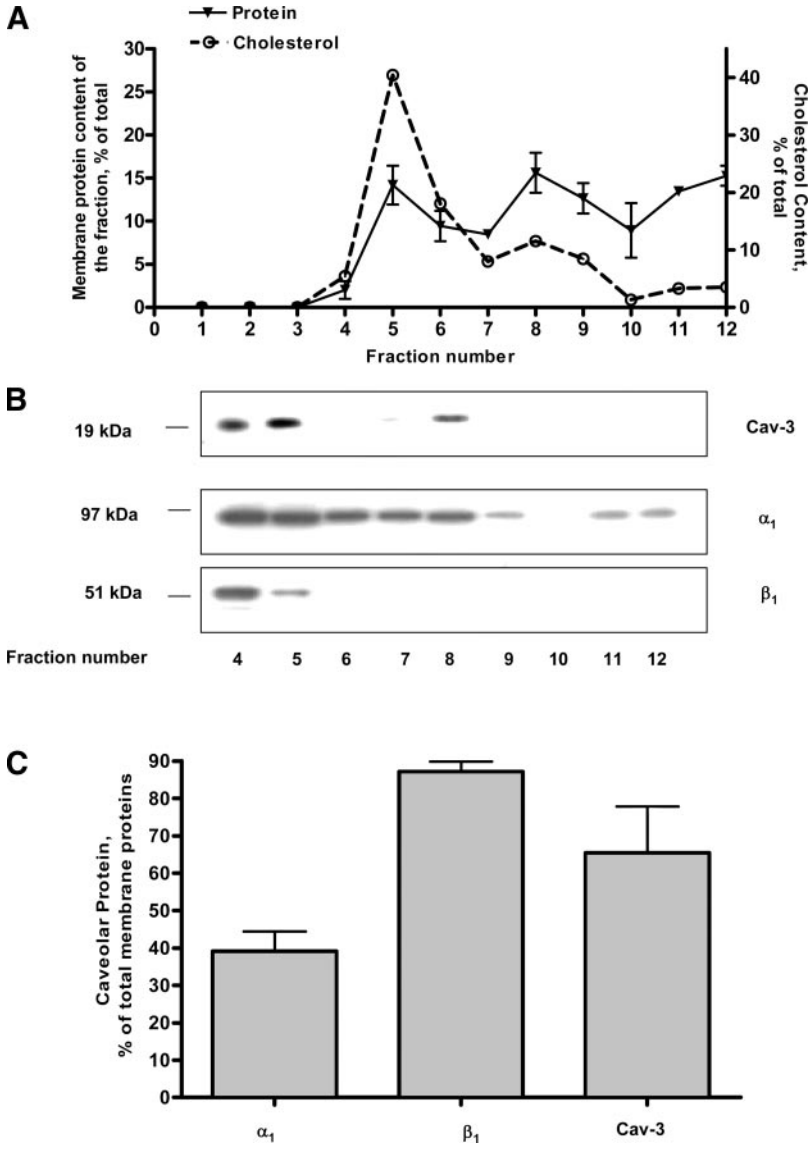

Fig. 5. Distribution of subunits of Na^+-K^+-ATPase and caveolin-3 in caveolar and noncaveolar membranes of adult cardiac myocytes prepared by a nondenaturing procedure. Cell lysates were fractionated by the KCl modification of the carbonate-based procedure as indicated in MATERIALS AND METHODS, and the resulting membrane fractions were assayed as indicated in Figs. 1 and 2. *A*: protein contents (means ± SE) of 3 fractionated myocyte preparations and cholesterol content of 1 fractionated preparation. *B*: typical blots of a fractionated preparation. *C*: %total contents of α_1, β_1, and caveolin-3 present in *fractions 4* and *5* (means ± SE, $n = 3$).

α_1/β_1 in rat kidney microsomes has been determined to be close to one (18). By procedures similar to those of the experiments of Fig. 2, we determined the relative α_1 and β_1 contents of different dilutions of rat kidney microsomes and crude cellular membranes of myocytes (MATERIALS AND METHODS) prepared from five different hearts. Assuming the molar ratio of one for kidney microsomes, we found the molar α_1/β_1 ratio for cardiac membranes to be 2.02 ± 0.28 (mean ± SE; $n = 5$). Based on this value and the data of Fig. 2*B*, it may easily be estimated that the molar α_1/β_1 ratio is about one in caveolae and about five in noncaveolar membranes. Evidently, in the latter, there is a large molar excess of α_1 over β_1.

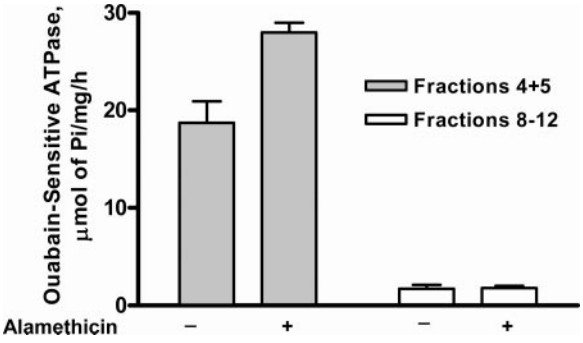

Fig. 6. Na^+-K^+-ATPase activities of the caveolar and noncaveolar membranes of adult cardiac myocytes prepared by the KCl procedure. Membranes were obtained from the fractionated lysates of 3 preparations and assayed as described in MATERIALS AND METHODS (means ± SE).

Interactions of caveolins with Na^+-K^+-ATPase. Caveolin-3 oligomerization is responsible for the formation of flask-shaped caveolar invaginations (33, 41), and heterooligomerization of caveolin-3 and caveolin-2 has also been demonstrated in skeletal muscle cells and neonatal cardiac myocytes (2, 35). To assess the possibility of interactions of the α,β-oligomers of Na^+-K^+-ATPase with the caveolin oligomers, coimmunoprecipitation studies were done. When neonatal myocytes were solubilized in a detergent mixture (octylglucoside and Triton X-100) that is known to preserve caveolin oligomers (2, 35, 41), and subjected to immunoprecipitation with an antibody specific to β_1-subunit, the Na^+-K^+-ATPase subunits and

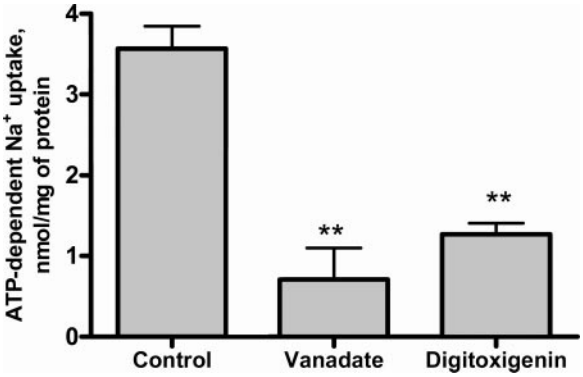

Fig. 7. Na^+ transport by Na^+-K^+-ATPase in sealed cardiac caveolar membranes. Caveolar membranes (*fractions 4* and *5*) obtained by the KCl procedure were assayed for ATP-dependent $^{22}Na^+$ uptake as described in MATERIALS AND METHODS (means ± SE; $n = 9$, control; $n = 3$, vanadate; $n = 4$, digitoxigenin). $^{**}P < 0.01$, relative to control.

caveolin-3 were detected in the precipitate (Fig. 8*A*). With the use of neonatal or adult myocyte lysates, or caveolar fractions isolated from adult myocytes, immunoprecipitation with an antibody specific for the caveolin-3 also resulted in the isolation of detergent-solubilized oligomers containing the β_1-subunit, caveolin-3, and caveolin-2 (Fig. 8*B*).

Distribution of α- and β-subunits in caveolar and noncaveolar membranes of cells other than adult cardiac myocytes. In neonatal rat cardiac myocytes, the relative distributions of the subunits in the membrane fractions (Fig. 9) were similar to those of the adult cardiac myocytes (Fig. 1). However, in two other tested cell types (A7r5 cells and adult rat cardiac fibroblasts), the β_1-subunit was not as restricted to caveolae as it is in cardiac myocytes (Fig. 9).

Relative distributions of α- and β-subunits in cardiac myocyte endosomes. Because different internalization pathways of the subunits of Na^+-K^+-ATPase may be responsible for their uneven distributions in the caveolar and noncaveolar membranes (see DISCUSSION), it was of interest to examine the relative levels of the subunits in endosomes. Using the same preparation of neonatal cardiac myocytes, we prepared caveolar membranes by the carbonate-based procedure and endosomal membranes by a procedure that is commonly used for the preparation of two membrane fractions that are enriched with early and late endosomes (11), and we subjected the three membrane fractions to Western analysis with antibodies against caveolin-3, the endosomal marker EEA1, and the α- and β-subunits (Fig. 10). The relative levels, per unit of protein, of caveolin-3 and EEA1 in the three membrane fractions were as expected (Fig. 10*A*). Importantly, the α/β ratio was clearly higher in late endosomes than in early endosomes or caveolae (Fig. 10*B*). These findings suggest different internalization paths of the two subunits.

DISCUSSION

The studies presented here confirm our previous findings showing that the isolated rat cardiac caveolar membranes contain less than half of the cellular content of the catalytic subunits (the α-subunits) of Na^+-K^+-ATPase but also demonstrate that the major cardiac β-subunit of the enzyme resides almost exclusively in caveolar membranes. In agreement with the established dependence of the catalytic activity on α,β-association (9, 16), we also find that most of the Na^+-K^+-ATPase activity of these cardiac myocytes is also concentrated in the caveolar membranes. Evidently, the caveolar inpocketings that constitute ~20% of cardiac myocyte plasma membrane surface (7, 19) are the major sites where the linked active transport of Na^+ and K^+ occurs.

Uneven distribution of the active enzyme among caveolar and noncaveolar membranes. To obtain functional Na^+-K^+-ATPase in caveolar and noncaveolar membranes, it was necessary to modify the widely used carbonate-based fractionation procedure by replacing Na_2CO_3 with KCl because of the carbonate-induced inactivation of the enzyme (Fig. 4). This finding is consistent with previous observations on the denaturation of Na^+-K^+-ATPase at highly alkaline pH (32). Replacement of Na_2CO_3 with NaCl has also been found useful for the detergent-free cell fractionation and preparation of caveolae where inactivation of a membrane receptor by carbonate has been observed (10). It is appropriate to note, however, that not all intrinsic membrane proteins are inactivated by high alkalinity (e.g., see Ref. 32).

What kind of cardiac membranes contain the enzyme subunits that are not in caveolae? Although the carbonate-based fractionation procedure, or its KCl modification, is suitable for the separation of low buoyant-density caveolar membranes from the heavier noncaveolar membranes, the latter fractions are clearly the unresolved mixtures of several cellular mem-

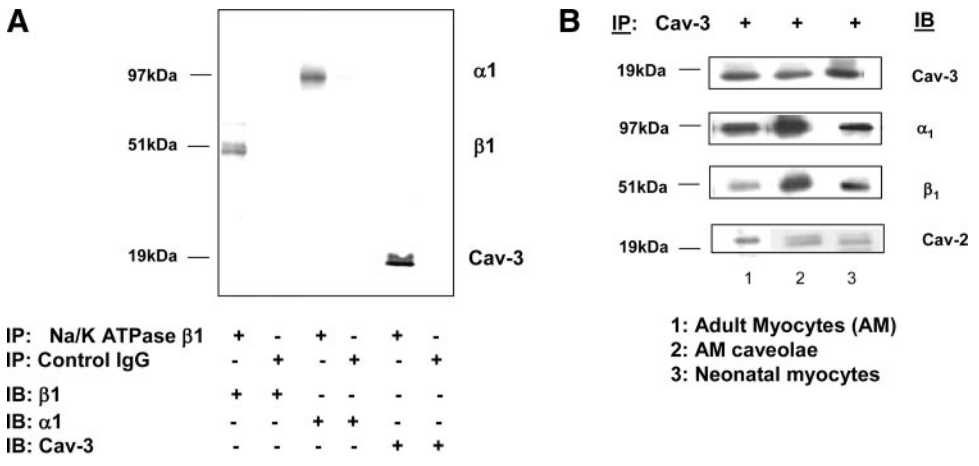

Fig. 8. Coimmunoprecipitation of Na^+-K^+-ATPase subunits and caveolins. Isolated adult cardiac myocytes, cultured neonatal myocytes, or caveolar membranes (*fractions 4* and *5*) prepared from adult myocytes were detergent solubilized, immunoprecipitated, and subjected to Western blot analysis as described in MATERIALS AND METHODS. *A*: neonatal lysates immunoprecipitated with anti-β_1 antibody. *B*: indicated lysates immunoprecipitated with anti-caveolin-3 antibody. Appropriate normal IgGs were used as negative controls (shown in *A* but not shown in *B*). Three separate experiments showed the same qualitative results. IP, immunoprecipitation; IB, immunoblot.

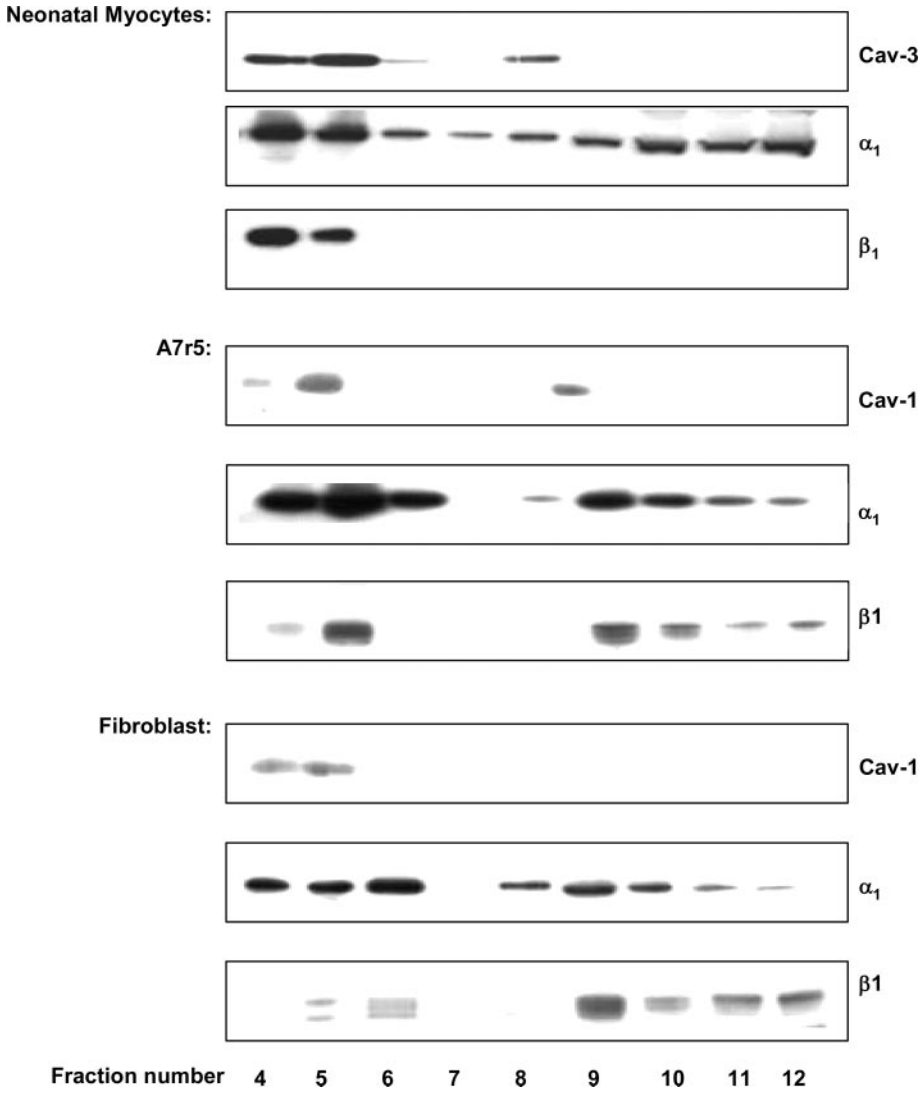

Fig. 9. Relative distribution of α_1- and β_1-subunits of Na^+-K^+-ATPase in caveolar and noncaveolar membranes of neonatal rat cardiac myocytes, A7r5 cells, and adult rat cardiac fibroblasts. Fractionations and assays were done as described in Fig. 1 and MATERIALS AND METHODS.

branes (Fig. 1). In spite of this heterogeneity, because Na^+-K^+-ATPase activity is detected in noncaveolar membranes that are clearly separated from caveolae (Fig. 6), properly folded and active α,β-oligomers must also exist in noncaveolar membranes. It is reasonable to assume that these active units are in the bulk plasma membrane and perhaps in Golgi and sarcoplasmic reticulum/endoplasmic reticulum where the α,β-assembly and maturation are known to occur (9, 16). That active enzyme is detectable in endoplasmic reticulum and Golgi has been shown in a heterologous expression system (8).

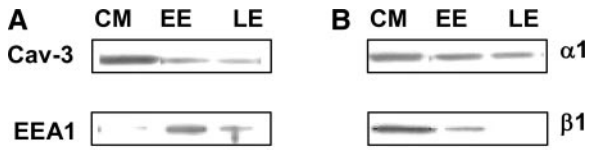

Fig. 10. Relative distributions of α_1- and β_1-subunits of Na^+-K^+-ATPase in caveolar membranes and endosomes of neonatal cardiac myocytes. Using the same myocyte preparation, caveolar membranes (CM, *fractions 4* and *5*) were prepared as described in Fig. 1, and early endosomes (EE) and late endosomes (LE) were prepared as indicated in MATERIALS AND METHODS. Equal amount of protein from each membrane fraction was used for immunoblots obtained with an indicated antibody. In a second experiment with a different myocyte preparation, the results were nearly identical to those shown.

The molar ratio of α/β seems to be close to one in caveolar membranes, but there is a large excess of α in noncaveolar membranes (see RESULTS). Because of the heterogeneity of the noncaveolar membrane fractions, it is not possible to say whether or not the excess α coexists with the active α,β-oligomers in the same membrane compartment. There are several prior observations indicating that, in different cell types, or different membrane pools, excess of one subunit or the other may exist (18 and references therein).

Origin of the uneven distributions of Na^+-K^+-ATPase subunits. If it is true, as most evidence for the assembly of oligomeric proteins indicates (13), that α and β associate early in the endoplasmic reticulum upon synthesis and membrane integration, and remain associated in the course of delivery to the plasma membrane (8, 9), how may the clear uneven occurrence of the two subunits in various membrane fractions be explained? A possibility that has been considered before is the independent synthesis and maturation of the unaccompanied subunit (18). An alternative is that the two subunits part

company on the return trip from the plasma membrane and its microdomains and move at different rates on their respective degradation pathways. It was for this reason that, in the exploratory experiments of Fig. 10, we compared the relative levels of α- and β-subunits in membrane fractions enriched with early and late endosomes. The apparent absence of β from the late but not the early endosomes and the presence of α in both suggest that the two subunits remain together in early recycling endosomes but not so in the late endosomes that lead to lysosomal degradation. Although this may not fit the classical pathway of clathrin-mediated endocytosis, we should note that there is now ample evidence for caveolae/raft-mediated endocytosis and that the two alternative pathways may merge by undetermined mechanisms, perhaps through caveosomes (27, 29, 40). That epithelial Na^+-K^+-ATPase is internalized through the clathrin-dependent route has been known for a long time (e.g., see Ref. 3), and recent evidence also suggests its internalization by a caveolin-dependent route (21). Clearly, further comparative studies on the degradation pathways and rates of the subunits of cardiac Na^+-K^+-ATPase are required. It is important to note, however, that mechanisms responsible for the uneven distribution of the subunits in various membranes must be highly cell specific, as evident by the present (Fig. 9) and previous findings (18).

Oligomeric state of the caveolar enzyme. The present findings suggest interactions between the α,β-subunits of cardiac Na^+-K^+-ATPase, caveolin-3, and caveolin-2 (Fig. 8). Whether these interactions are direct or through other proteins within a detergent-resistant assembly cannot be determined by the immunoprecipitation experiments. There is extensive previous evidence indicating that, in caveolae microdomains, caveolin-3 and caveolin-2 (or caveolin-1 and caveolin-2) exist as large oligomers in association with other proteins (33, 41). In epithelial caveolae, interaction of the α-subunit of Na^+-K^+-ATPase with caveolin-1 and other caveolar-signaling proteins has been shown recently (43, 48). There is also a large body of evidence, accumulated over decades, to indicate that the minimum quaternary structure of Na^+-K^+-ATPase is a tetramer of the α,β-dimer (31, 42). Taking these facts together, we consider it most likely that cardiac caveolar Na^+-K^+-ATPase exists as a large aggregate of Na^+-K^+-ATPase subunits, caveolins, and related signaling proteins. In spite of the complexity of this assembly, it is evident that at least some of its Na^+-K^+-ATPase subunits are capable of performing the essential ion-pumping function of the enzyme. Studies are in progress to determine whether or not there are significant differences between the catalytic and transport functions of caveolar and noncaveolar pools of Na^+-K^+-ATPase.

Functional implications. The preponderance of the pumping α,β-oligomers in cardiac caveolae, relative to other cardiac membranes, strengthens our previous suggestion that these microdomains are the sites of initiation of digitalis-induced signaling, leading to transcriptional regulation of growth-related genes, stimulation of protein synthesis, and myocyte hypertrophy (23, 44). Although the initial proposal (23) was based on the mere caveolar presence of Na^+-K^+-ATPase subunits, and their resulting proximities to a host of caveolar signaling partners, the present data indicating that cardiac caveolae are the major portals of active Na^+-K^+ fluxes lend more credibility to the hypothesis that these inpocketings of the sarcolemma/T tubules are indeed responsible for the spacial integration of the pumping and signaling functions of Na^+-K^+-ATPase. Considering this, and the well-established fact that changes in the active Na^+-K^+ fluxes of cardiac myocytes are linked to altered intracellular Ca^{2+} concentration and contractility (30, 44), the question arises as to whether cardiac caveolae are also the sites for the integration of the multiple function of Na^+-K^+-ATPase with those of the cardiac Ca^{2+}-transporting proteins. This is likely because of a larger body of experimental evidence pointing to the role of caveolae of different cell types, including myocytes, in the regulation of Ca^{2+} entry in cells and Ca^{2+}-dependent signaling (15). However, because of the apparent existence of different cargo-specific populations of cardiac caveolae (36), it remains to be determined if Na^+-K^+-ATPase and related Ca^{2+}-regulatory proteins reside in the same cardiac caveolae.

Our limited experiments on different cell types (Fig. 9) suggest that the heavy concentration of the ion pumping Na^+-K^+-ATPase in caveolar membranes may be specific to cardiac myocytes. Although the cause(s) of the cell specificity of this phenomenon remains to be established, it is appropriate to comment on its functional implications. Studies of the recent past on the signal-transducing function of Na^+-K^+-ATPase have clearly shown that, although there are similarities in some early stimulus-induced signaling events detected in different cells (e.g., transactivations of Src and EGFR), there is also significant diversity in the nature of activated signaling pathways and the downstream consequences (e.g., inhibition or activation of growth) in various cell types (22, 44). Some of the potential causes of this diversity have been discussed before (44). An additional possibility is that caveolar and noncaveolar pools of the plasma membrane Na^+-K^+-ATPase may both be involved in stimulus-induced signaling via transactivations of different signaling partners and that the relative distribution of Na^+-K^+-ATPase in the two pools contributes to the diversity of the downstream events.

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