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Role of caveolae in signal-transducing function of cardiac Na^+/K^+-ATPase

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Liu, Lijun, Kamiar Mohammadi, Behrouz Aynafshar, Haojie Wang, Daxiang Li, Jiang Liu, Alexander V. Ivanov, Zijian Xie, and Amir Askari. Role of caveolae in signal-transducing function of cardiac Na^+/K^+-ATPase. *Am J Physiol Cell Physiol* 284: C1550–C1560, 2003. First published February 26, 2003; 10.1152/ajpcell.00555.2002.—Ouabain binding to Na^+/K^+-ATPase activates Src/epidermal growth factor receptor (EGFR) to initiate multiple signal pathways that regulate growth. In cardiac myocytes and the intact heart, the early ouabain-induced pathways that cause rapid activations of ERK1/2 also regulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and contractility. The goal of this study was to explore the role of caveolae in these early signaling events. Subunits of Na^+/K^+-ATPase were detected by immunoblot analysis in caveolae isolated from cardiac myocytes, cardiac ventricles, kidney cell lines, and kidney outer medulla by established detergent-free procedures. Isolated rat cardiac caveolae contained Src, EGFR, ERK1/2, and 20–30% of cellular contents of α_1- and α_2-isoforms of Na^+/K^+-ATPase, along with nearly all of cellular caveolin-3. Immunofluorescence microscopy of adult cardiac myocytes showed the presence of caveolin-3 and α-isoforms in peripheral sarcolemma and T tubules and suggested their partial colocalization. Exposure of contracting isolated rat hearts to a positive inotropic dose of ouabain and analysis of isolated cardiac caveolae showed that ouabain caused *1*) no change in total caveolar ERK1/2, but a two- to threefold increase in caveolar phosphorylated/activated ERK1/2; *2*) no change in caveolar α_1-isoform and caveolin-3; and *3*) 50–60% increases in caveolar Src and α_2-isoform. These findings, in conjunction with previous observations, show that components of the pathways that link Na^+/K^+-ATPase to ERK1/2 and $[Ca^{2+}]_i$ are organized within cardiac caveolae microdomains. They also suggest that ouabain-induced recruitments of Src and α_2-isoform to caveolae are involved in the manifestation of the positive inotropic effect of ouabain.

digitalis; heart failure; rafts; sodium pump; Na^+/Ca^{2+} exchanger

THE ENERGY-TRANSDUCING ion pump Na^+/K^+-ATPase maintains the normal gradients of Na^+ and K^+ across the plasma membrane of most eucaryotic cells (34). It has recently been shown that in cardiac myocytes and several other cell types, Na^+/K^+-ATPase also acts as a signal transducer; i.e., in response to ouabain and related cardiac glycosides, the enzyme interacts with neighboring membrane proteins to relay messages to intracellular signaling complexes, the mitochondria, and the nucleus (45). The most proximal ouabain-induced interaction seems to be between Na^+/K^+-ATPase and Src, leading to Src activation and epidermal growth factor receptor (EGFR) transactivation (7, 8), activations of protein kinase C (PKC) and the Ras/Raf/MEK/ ERK1/2 cascade (13, 25, 26), and a host of subsequent downstream effects emanating from these rapid proximal signaling events. To date, the downstream consequences of ouabain-induced signaling through Na^+/K^+-ATPase have been studied in some detail only in cardiac myocytes, where ouabain and related cardiac glycosides exert their well-known effects on cardiac contractility. In these cells, such consequences include increased mitochondrial generation of reactive oxygen species, activation of transcription factors activator protein-1 and nuclear factor-κB, transcriptional regulation of early- and late-response growth-related genes, increased rate of protein synthesis, and myocyte hypertrophy (45). Recent evidence (41) indicates that the rapid ouabain-induced signaling that leads to the activation of ERK1/2 is also essential to the manifestation of the classical effects of ouabain on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cardiac contractility; i.e., the positive inotropic effect of the drug.

It is generally accepted that the plasma membrane contains microdomains (rafts) that are rich in cholesterol and sphingolipids relative to the remainder of the plasma membrane and that caveolae are specific forms of rafts that contain the marker proteins caveolins (1, 30, 35). Because such microdomains have been implicated as sites of assembly and regulation of signaling complexes associated with a variety of plasma membrane receptors (1, 30, 35), we were prompted to explore their possible involvement in the newly appreciated signal-transducing functions of Na^+/K^+-ATPase. Specifically, the primary goals of this study were twofold. First, in view of the ambiguities of previous reports on the caveolar localization of Na^+/K^+-ATPase, we wanted to determine whether or not this enzyme is a normal resident of caveolae. Our findings establish that it is. Second, because of the central role of ERK1/2

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in the manifestation of ouabain effects on cardiac myocytes and the intact heart (26, 45), we assessed whether or not the caveolar pool of cardiac Na^+/K^+-ATPase isoforms are involved in the ouabain-induced activation of signal pathways that link Na^+/K^+-ATPase to ERK1/2 concomitant with the development of the positive inotropic effect of ouabain on the heart.

MATERIALS AND METHODS

Materials. Chemicals of highest purity and culture media were purchased from Sigma (St. Louis, MO). Anti-caveolin-3 monoclonal antibody (clone 26), and anti-caveolin-1 monoclonal antibody (clone C060) were obtained from BD Transduction Laboratories (Lexington, KY). Anti-caveolin-3 polyclonal antibody was purchased from Affinity BioReagents (Golden, CO). Dynabeads coated with goat anti-mouse IgG were obtained from Dynal (catalog no. M-450; Lake Success, NY). Anti-Na^+/K^+-ATPase α_1 monoclonal antibody, anti-Na^+/K^+-ATPase α_2 polyclonal antibody, anti-Na^+/K^+-ATPase β_1 monoclonal antibody, anti-Src (clone GD11) monoclonal antibody, polyclonal anti-EGFR antibody, and rabbit anti-sheep secondary antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies against ERK1/2, phosphorylated ERK1/2, PKA catalytic subunit, goat anti-rabbit secondary antibody, and goat anti-mouse secondary antibody were obtained by Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against Na^+/K^+-ATPase α_1 (α6F) was obtained from Developmental Studies Hybridoma Bank, The University of Iowa (Iowa City, IA). Anti-Na^+/K^+-ATPase α_3 polyclonal antibody against a synthetic peptide corresponding to the NH_2 terminus of the rat subunit was a gift from Dr. R. W. Mercer (Washington University, St. Louis, MO). Peptide *N*-glycosidase F was purchased from New England Biolabs (Beverly, MA). Kidney medullas were obtained by dissection from frozen kidneys. Pig kidneys and beef hearts were obtained from slaughterhouses. Rats used as sources of organs were housed and euthanized according to institutional policies.

Cell preparation and culture. Neonatal rat cardiac myocytes were cultured from the ventricles of 1- to 2-day-old rats, as described previously (13, 25), and used after 24 h of serum starvation. Calcium-tolerant adult rat cardiac myocytes were also prepared as described before (41). Freshly made suspensions were either used for caveolae isolation or seeded on laminin-coated coverslips and used for immunocytochemistry. Pig kidney LLC-PK_1 cells and human transformed primary embryonic kidney cells (HEK-293) (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. When 80–90% confluence was reached, cells were serum starved for 24 h and used for caveolae preparation from cell lysates.

Ouabain-induced positive inotropy in isolated hearts. Conventional isolated Langendorff preparations of rat heart were set up for the measurement of cardiac contractility and perfused with normal Krebs-Henseleit solution as described before (26). Each heart was perfused for 30 min with the control buffer before contractility measurements were recorded. To induce the effects of ouabain, the perfusion solution was switched to the same buffer containing 50 μM ouabain. As shown before (26), this dose of ouabain causes a significant and sustained doubling of the rate of left ventricular pressure increase without producing toxic arrhythmias. After 10 min of perfusion, when the peak positive inotropic effect of ouabain was obtained (26), hearts were quick-frozen in liquid nitrogen and lysates of powdered ventricular samples were used for caveolae preparation.

Fractionation of cell/tissue lysates for caveolae preparation by carbonate-based procedure. This procedure was done by slight modification of the procedure of Song et al. (37). A sample of intact cells (myocytes, LLC-PK_1 cells, and HEK-293 cells) or tissue (cardiac ventricle, kidney outer medulla) with a total protein content of 8–16 mg was placed at 4°C in 2 ml of 0.5 M Na_2CO_3 solution (pH 11) containing 1 mM EDTA, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 1 mM NaF, 10 nM okadaic acid, 10 μg/ml aprotonin, and 10 μg/ml leupeptin. In specified experiments, this solution also contained 1% Triton X-100. All subsequent steps were done on ice or at 4°C. The sample was homogenized at *setting 5* of a Polytron homogenizer (three 6-s bursts) and sonicated at *setting 3* of a Branson Sonifier model 250 (three 40-s bursts). We added 2 ml of 90% sucrose to the homogenate, prepared in 25 mM MES (pH 6.5) plus 150 mM NaCl (MBS). This suspension was placed in the bottom of the centrifuge tube and was overlaid with 4 ml of 35% sucrose and then 4 ml of 5% sucrose, each prepared in MBS containing 250 mM Na_2CO_3. The sample was centrifuged with a rotor (model SW41, Beckman) at 39,000 revolutions/min for 17–18 h, and twelve 1-ml fractions (numbered from top to bottom) were collected.

Preparation of caveolae from cardiac sarcolemma by OptiPrep procedure. Highly purified cardiac sarcolemma were prepared from bovine or rat heart ventricles, following the method of Jones (11). A sample of these plasma membranes (0.7–0.8 mg total protein) was suspended in 2 ml of 0.25 M sucrose, 1 mM EDTA, and 20 mM Tricine (pH 7.6) and sonicated on ice with three series of two consecutive bursts, 6–8 s each, at *setting 4* of a Branson Sonifier 250, with 2-min intervals between the series. The sample was then fractionated on OptiPrep gradients by the procedures of Smart et al. (36), as subsequently modified (42). The opaque band at the 5% OptiPrep overlay after the second fractionation step was designated as caveolae.

Immunoblot analysis. Samples were subjected to 10 or 12% SDS-PAGE, transferred to nitrocellulose membrane, and probed with appropriate antibodies by standard procedures. The immunoreactive bands were developed and detected using enhanced chemiluminescence. For quantitative comparisons, images were scanned with a densitometer. Different dilutions of samples were subjected to SDS-PAGE, and multiple exposures of the films were used to ensure that quantitations were made within the linear range of the assays. Quantitative comparisons of blots that are not subjected to such procedures may be misleading.

Immunofluorescence microscopy. Myocytes attached to coverslips were washed two times in phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-Ca-Mg) and fixed in −20°C methanol for 10 min. Cells were rinsed one time in PBS-Ca-Mg, incubated for 15 min in cell permeabilization buffer (PBS-Ca-Mg, 0.3% Triton X-100, and 0.1% bovine serum albumin), and then incubated in goat serum dilution buffer (GSDB; 16% filtered goat serum, 0.3% Triton X-100, 20 mM sodium phosphate, pH 7.4, and 150 mM NaCl) for 30 min at room temperature to block nonspecific IgG binding sites. Myocytes were incubated overnight at 4°C with anti-Na^+/K^+-ATPase α_1 monoclonal antibody (1:100) and anti-caveolin-3 polyclonal antibody (1:100) in GSDB or anti-Na^+/K^+-ATPase α_2 polyclonal antibody (1:100) and anti-caveolin-3 monoclonal antibody (1:100) in GSDB, respectively. The same procedure was also performed by using normal goat serum or PBS instead of primary antibody as a negative control, to ensure the specificity of the double-staining procedure and the specificity of fluorescent second-

ary antibodies. On the following day, myocytes were washed three times for 5 min each in permeabilization buffer and then incubated for 2 h at room temperature with Alexa Fluor 488-conjugated anti-mouse IgG(H+L) and Alexa Fluor 546-conjugated anti-rabbit IgG(H+L) antibodies (Molecular Probes, Eugene, OR) in GSDB or with Alexa Fluor 488-conjugated anti-rabbit IgG(H+L) and Alexa Fluor 546-conjugated anti-mouse IgG (H+L) antibodies in GSDB, respectively. Myocytes were washed three times for 5 min each in permeabilization buffer and once in 10 mM sodium phosphate (pH 7.5). The coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were acquired by confocal laser scanning microscope (Bio-Rad Radiance 2000) with the use of the 488- and 543-nm lines of Ar-Kr and He-Ne lasers. An Olympus Uplan-Apo water-immersion objective ×60/1.2 NA was used, and the software LaserSharp 2000 (Bio-Rad) was used for image acquisition, storage, and visualization.

Immunoaffinity isolation of caveolae. This was done by modification of previously described procedures (38, 43). Caveolae samples isolated by the detergent-free carbonate-based procedure were precleared with Dynal M450 beads coated with goat anti-mouse IgG. Additional Dynabeads were incubated with anti-caveolin-3 monoclonal antibody overnight at 4°C, and the cleared sample was then added and incubated for another 1 h at 4°C. Immune complexes were collected by magnetic separation and washed four times in 20 mM Tris·HCl (pH 7.4). Two fractions, material bound to beads (bound) and material not bound to beads (unbound), were subjected to SDS-PAGE and Western analysis.

Other assays. Na^+/K^+-ATPase activity was assayed (46) at 37°C by measuring the initial rate of release of ^{32}Pi from [γ-^{32}P]ATP in a medium containing 100 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, and 20 mM Tris·HCl (pH 7.4). Each assay was done in the presence and absence of 1 mM ouabain to assess the ouabain-sensitive component of the activity. Protein was determined by the Bio-Rad DC protein colorimetric assay.

Analysis of data. 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

Colocalization of Na^+/K^+-ATPase subunits and related signaling proteins in cardiac caveolae. Several previous studies (5, 9, 18, 33, 48) had concluded that caveolae/rafts prepared from several cell types, including cardiac myocytes, do not contain Na^+/K^+-ATPase. Because our preliminary experiments suggested otherwise, we set out to resolve this issue. First, we focused on cardiac caveolae because most of our studies on the signal-transducing role of Na^+/K^+-ATPase had been done in cardiac myocytes and the intact heart (26, 45). The widely used detergent-free and carbonate-based procedure of Song et al. (37) had been used successfully in numerous studies on cardiac caveolae prepared from cultured cardiac myocytes and ventricles (4, 5, 27, 28, 31, 32). Hence, we applied this density gradient fractionation procedure to homogenized/sonicated samples of rat heart ventricles, purified adult rat heart myocytes, and cultured neonatal rat cardiac myocytes. The fractions were then assayed for protein content and subjected to Western blot analyses for the muscle-specific caveolin-3 and the other indicated proteins. Typical distribution patterns of total protein and caveolin-3 for a fractionated sample of ventricle are shown in Fig. 1. Nearly identical patterns were obtained after fractionations of the adult or neonatal myocyte preparations. These findings are in agreement with previous observations on cardiac preparations fractionated by the same procedure (4, 5, 28, 32), showing that most of the caveolin-3 content of the homogenate is located in light fractions (*fractions 4* and *5*) that contain <3% of the total protein. Such cardiac light fractions that contain caveolin-3, and are prepared by a procedure similar to that used here, have been shown (32) by electron microscopic examination to contain vesicles and membrane fragments that resemble the "cave-like" caveolae vesicles that are noted on the plasma membrane of the intact cardiac cells (6, 16).

The α_1-isoform of the catalytic subunit of Na^+/K^+-ATPase is the predominant isoform (~80% of the total α-content) of the adult rat heart and the rat cardiac myocytes (20, 22, 23, 39). Typical blots of the α_1-subunit for the fractionated samples of ventricles and adult myocytes are shown in Fig. 1. These blots were obtained by using an equal amount of protein from each fraction and equal film exposure time for image development from each fraction. Considering this, and the pattern of distribution of total protein in the 12 fractions (Fig. 1, *top*), we may reach the following semiquantitative conclusions: *1*) most of the α_1 content of the fractionated lysate is recovered in heavy *fractions 8–12*; *2*) the caveolin-3-rich light *fractions 4* and *5* also contain significant quantities of the α_1-subunit; and *3*) per-unit protein *fractions 4* and *5* are enriched in α_1 relative to heavy *fractions 8–12*.

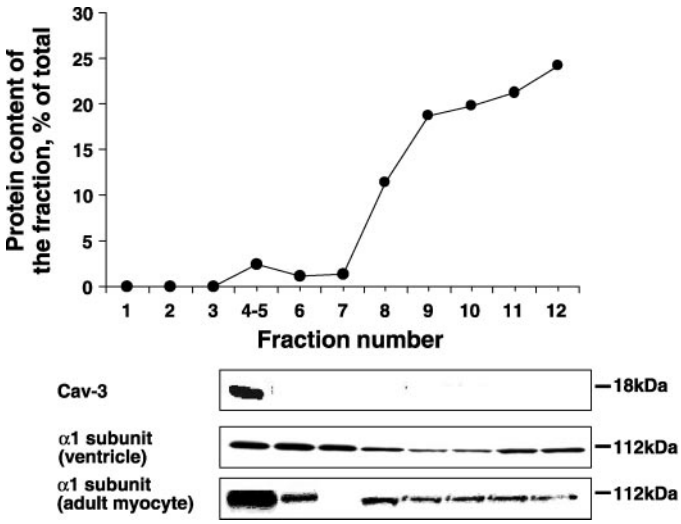

Fig. 1. Localization of the α_1-subunit of Na^+/K^+-ATPase in caveolin-3 (Cav-3)-rich domains of rat heart ventricles and adult myocytes. Samples of lysates (8–16 mg of protein) were fractionated by the detergent-free carbonate-based procedure, and 12 fractions of equal volume (1 ml each) were collected (see MATERIALS AND METHODS). Each fraction was assayed for protein, and an equal amount of protein from each fraction was subjected to SDS-PAGE and Western blot analysis with specific antibodies. The protein distribution pattern and the Cav-3 blots were similar for the samples from the two sources. *Fractions 1–3* did not contain protein.

In Fig. 2, several representative blots of a number of other proteins of interest in the fractionated samples of the cardiac preparations are presented. The results show that like the α_1-isoform, the less abundant cardiac isoforms of the rat (adult α_2 and neonatal α_3), Src, and ERK1/2 are also enriched per unit protein in caveolar fractions. The blot on the catalytic subunit of protein kinase A (Fig. 2) exemplifies the fact that not all fractionated proteins are enriched in the light fractions obtained by this procedure.

We used immunostaining and confocal microscopy to explore the subcellular localizations of caveolin-3 and the α_1 and α_2 isoforms of Na^+/K^+-ATPase in adult cardiac myocytes. In agreement with previous observations (23), the α-subunits were clearly localized in peripheral sarcolemma, T tubules, and intercalated disks (Fig. 3). Caveolar vesicles have been noted before by electron microscopic examination of the myocardium on both peripheral plasma membrane and T tubules (6, 16). Immunostaining also showed the presence of caveolin-3 in peripheral sarcolemma, T tubules, and intercalated disks (Fig. 3). Significantly, our findings indicated considerable overlap in the immunostaining of the α-subunits and caveolin-3 (Fig. 3). The combined data of Figs. 1–3, in conjunction with previous findings, strongly support the colocalization of caveolin-3 and a significant portion of Na^+/K^+-ATPase in cardiac caveolae.

Although the experiments of Figs. 1 and 2 showed the presence of significant amounts of Na^+/K^+-ATPase α-isoforms and several related signaling proteins in cardiac caveolae isolated from lysates, it was necessary to make a better quantitative assessment of the relative distributions of the proteins between caveolae and the remaining cellular compartments. Such determinations, based on single composite blots (e.g., those of Figs. 1 and 2), may be misleading because the relative intensities of bands in such blots are often over- or underestimated. It is therefore necessary to subject different dilutions of the various fractions to immunoblot analysis, and to quantitate multiple exposures of the luminescent images, to ensure the appropriate comparison of the contents of the various fractions. This procedure was done on each protein (shown in Fig. 4) by using ventricular samples from four hearts. After fractionation of each lysate sample by the carbonate-based procedure, and the assay of total protein content of each fraction, the content of the immunoreactive protein in each fraction was determined by procedures that optimize such comparative quantitations (as indicated above and in MATERIALS AND METHODS) and expressed in arbitrary relative units. Caveolar content of each protein, as a percentage of the total, was then calculated. The results of these experiments as summarized in Fig. 4 show the caveolar contents of caveolin-3, α_1, α_2, Src, ERK1, and ERK2. These data indicate that ~20–30% of total cellular contents of α_1- and α_2-isoforms are located in caveolae. The remainder must be in the bulk plasma membrane and the internal membranes. In skeletal muscle myocytes, it has been estimated that about half of the cellular content of Na^+/K^+-ATPase is in the plasma membrane and the other in the internal membranes (44). If we assume about the same distribution for rat cardiac myocytes, the data of Fig. 4 would suggest that 40–60% of the total plasma membrane pool is in caveolae and the remainder in the rest of the bulk plasma membrane. Because quantitative analysis of electron micrographs has suggested that caveolae microdomains constitute ~20–30% of the plasma membrane area of myocytes of the adult heart (6, 16), we may conclude tentatively that the α-subunits of cardiac Na^+/K^+-ATPase are concentrated in the caveolae microdomains relative to the remaining areas of the cardiac plasma membrane.

Caveolae preparations may be mixed with low buoyant density noncaveolar rafts (43). Using the approach of Stan et al. (38), which involved the isolation of caveolae on anticaveolin-coated magnetic beads, we sedimented/purified the cardiac caveolae on anticaveolin-3-coated beads. The results shown in Fig. 5 indicate that the subunits of Na^+/K^+-ATPase (α_1 and β_1), Src, and EGFR are indeed colocalized in the same microdomains that contain caveolin-3. We verified that the band reacting with the monoclonal anti-β_1 antibody is, indeed, the β_1-subunit by performing experiments showing that incubation of caveolae with *N*-glycosidase F converted the band to one with mobility well in accord with that of the unglycosylated β_1 protein (Fig. 5).

Caveolin-containing membranes of low buoyant density may also be obtained by fractionation of homogenates in the presence of Triton X-100, because of the

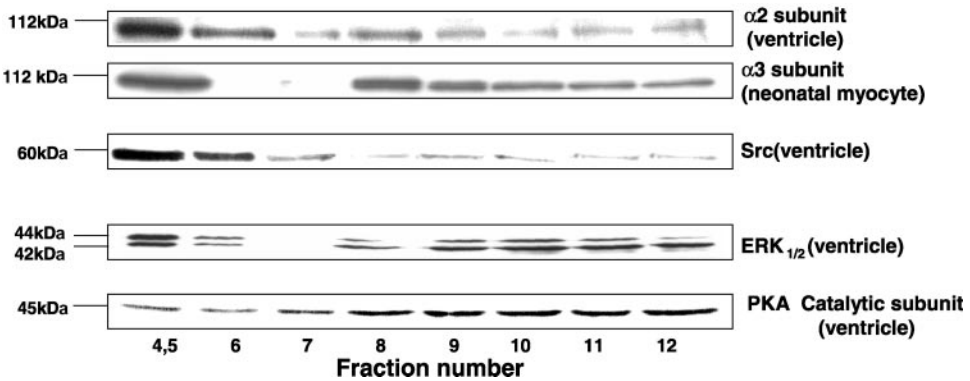

Fig. 2. Localization of the α_2- and α_3-isoforms of Na^+/K^+-ATPase, Src, and ERK1/2 in Cav-3-rich domains of cardiac ventricles and neonatal myocytes. Fractionations and immunoblots were performed as indicated in Fig. 1 and MATERIALS AND METHODS. PKA, protein kinase A.

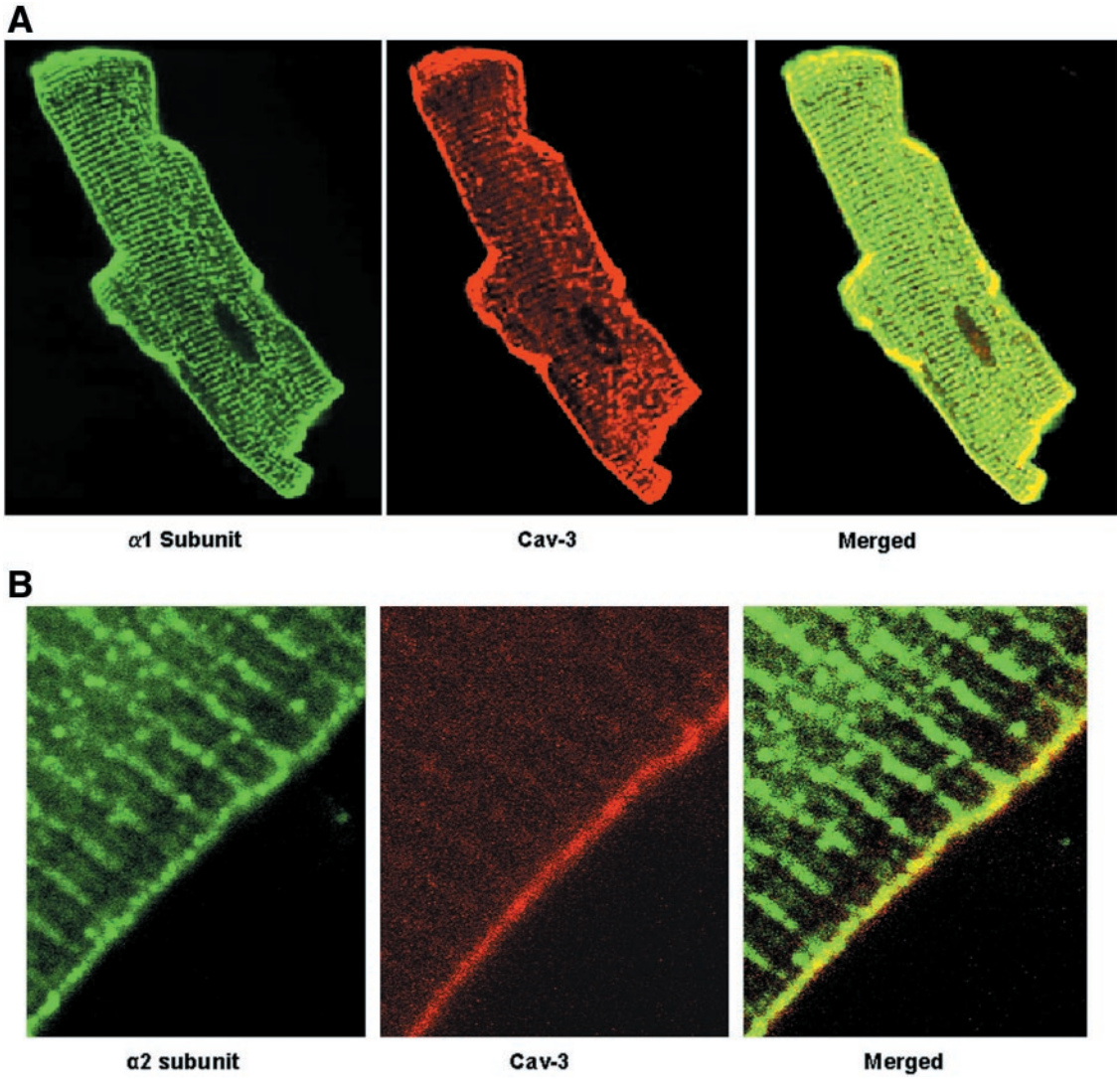

Fig. 3. Colocalization of Na^+/K^+-ATPase α-isoforms and Cav-3 in adult cardiac myocytes. Cultured myocytes were fixed for immunostaining and confocal microscopy (see MATERIALS AND METHODS). *A*: staining of α_1 (green) and Cav-3 (red); *B*: staining of α_2 (green) and Cav-3 (red) in greater detail.

insolubility of caveolins in this detergent at 4°C (1, 36). It is now widely recognized, however, that detergents may remove some caveolar proteins (1, 36, 37). When cardiac caveolae were prepared by the carbonate-based procedure in the presence and absence of 1% Triton X-100, comparable levels of caveolin-3 were detected in both preparations as expected, but Na^+/K^+-ATPase subunits were absent from the detergent-treated caveolae (Fig. 6). This explains why some studies (9, 18, 33), in which Triton X-100 was used, failed to detect Na^+/K^+-ATPase subunits in caveolae (see DISCUSSION).

Another widely used detergent-free procedure for the preparation of caveolae involves the fractionation of sonicated plasma membrane preparations on OptiPrep gradients (36, 42). We deemed it necessary, therefore, to test for the presence of Na^+/K^+-ATPase in cardiac caveolae prepared by this procedure. Immunoblots of such caveolae made from the plasma membranes of rat and bovine cardiac ventricles, prepared as described in MATERIALS AND METHODS, showed the presence of Na^+/K^+-ATPase subunits along with caveolin-3 (not presented).

Showing the presence of Na^+/K^+-ATPase subunits in caveolae does not establish that this pool is catalytically competent, especially because the lipid composition of the caveolae is known to be significantly different from that of the bulk plasma membrane (1, 35), and because Na^+/K^+-ATPase activity is known to be dependent on the nature of phospholipids and the level of membrane cholesterol (3, 34, 49). It was important, therefore, to know whether the Na^+/K^+-ATPase subunits detected in caveolar fractions exhibit enzyme activity. Caveolae prepared by the carbonate-based procedure are not suitable for the assay of Na^+/K^+-ATPase activity due to the high alkalinity of the preparative medium and its content of vanadate and fluoride (see MATERIALS AND METHODS), all of which have inhibitory effects on this activity. However, having

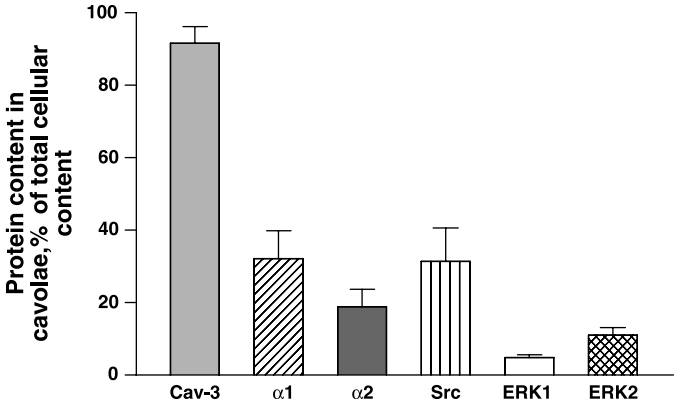

Fig. 4. Relative distributions of Cav-3, α_1- and α_2-isoforms of Na^+/K^+-ATPase, Src, and ERK1/2 in caveolar and noncaveolar fractions of cardiac ventricle lysates. Fractionations were performed as in Fig. 1 on samples from 4 different hearts. Immunoblots of the indicated proteins were obtained from each of the 12 fractions under conditions that are optimal for the quantitation of such blots, as described in MATERIALS AND METHODS. On the basis of these determinations and the assay of total protein content of each fraction, the content of each indicated protein in *fractions 4* and *5*, relative to total sample content, was calculated. Values are means ± SE (n = 4).

established that the OptiPrep-containing media do not inhibit Na^+/K^+-ATPase (not shown), we were able to detect ouabain-sensitive ATPase activity in cardiac caveolae prepared by the OptiPrep procedure (Fig. 7). We also compared this activity and the α-subunit content of caveolae with those of the sarcolemma used for the preparation of caveolae. The α-subunit was enriched in caveolae relative to sarcolemma, but the specific activity of caveolar ouabain-sensitive activity was lower than that of sarcolemma (Fig. 7). The cause of this remains to be determined (see DISCUSSION).

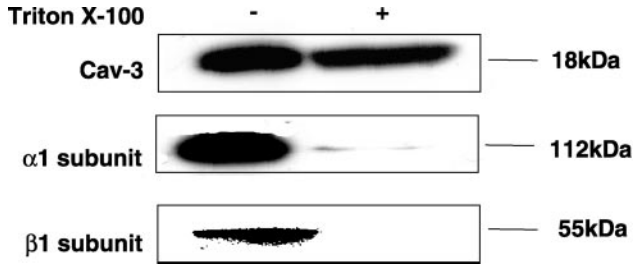

Fig. 6. Effects of Triton X-100 on the caveolar contents of Cav-3 and Na^+/K^+-ATPase subunits. Cardiac ventricular samples were fractionated by the carbonate-based procedure in the presence and absence of Triton X-100 (see MATERIALS AND METHODS). The combined *fractions 4* and *5* were immunoblotted for the indicated proteins.

Caveolar localization of Na^+/K^+-ATPase in cells other than cardiac myocytes. To determine whether the caveolar localization of Na^+/K^+-ATPase is a peculiarity of cardiac myocytes, we used the carbonate-based procedure to fractionate samples of LLC-PK_1 cells, HEK-293 cells, and the outer medullas of rat kidney and pig kidney. Fraction immunoblots (see Fig. 8) for HEK-293 cells and the pig kidney outer medulla clearly indicate the presence of Na^+/K^+-ATPase subunits and Src in the light fractions containing caveolin-1. As expected, no muscle-specific caveolin-3 was detected in these preparations (not shown). In these experiments, like those with cardiac preparations, Na^+/K^+-ATPase subunits were not as restricted to the light fractions as caveolin (Fig. 8). The results with LLC-PK_1 cells and the rat kidney medulla (not shown) were similar to those of Fig. 8, *A* and *B*, respectively, demonstrating the presence of significant amounts of Na^+/K^+-ATPase subunits in caveolin-containing light *fractions 4* and *5*.

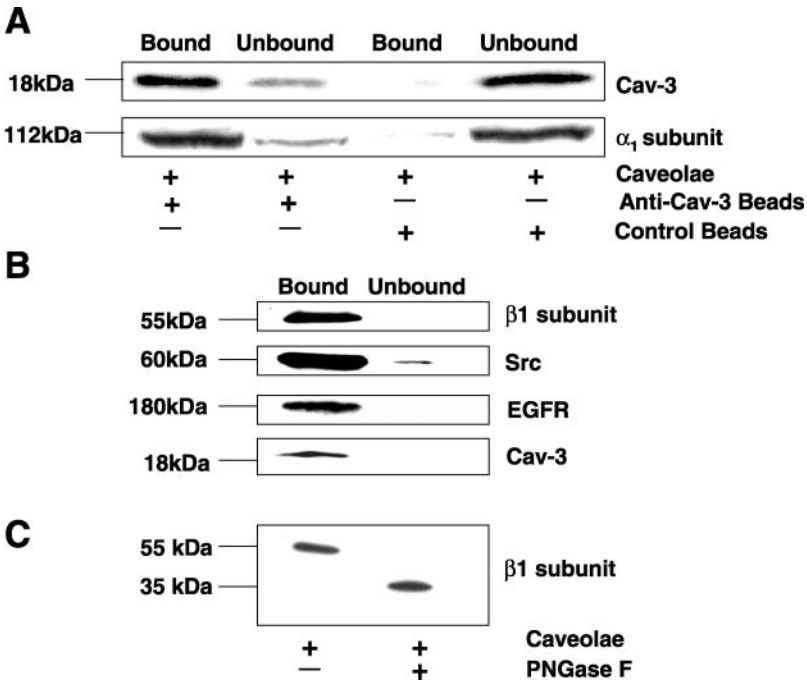

Fig. 5. Localization of Na^+/K^+-ATPase subunits (α_1 and β_1), Src, and epidermal growth factor receptor (EGFR) in caveolae that are immunoseparated from possible contaminating rafts. *A* and *B*: caveolae (*fractions 4* and *5*) prepared from cardiac ventricles (see Fig. 1) were purified on anti-Cav-3-coated magnetic beads (see MATERIALS AND METHODS) and immunoblotted for the indicated proteins. In *B*, the results with control beads, which were similar to those in *A*, are not shown. *C*: caveolae were treated with peptide *N*-glycosidase F (PNGase F) (40) before being subjected to immunoblot analysis with monoclonal anti-β_1 antibody.

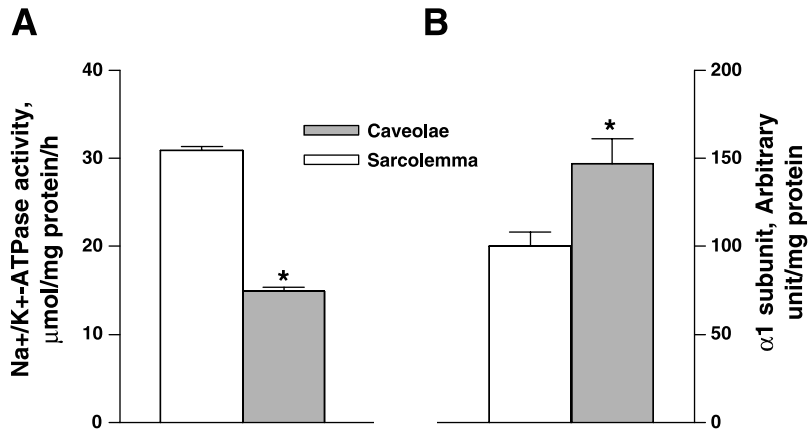

Fig. 7. Na^+/K^+-ATPase activities (*A*) and the α_1-subunit contents (*B*) of cardiac sarcolemma and caveolae prepared from the sarcolemma by the OptiPrep procedure. Partially purified sarcolemma were prepared from bovine heart ventricles and fractionated by the OptiPrep procedure to obtain a light caveolae fraction (see MATERIALS AND METHODS). Multiple caveolae preparations from the same batch of sarcolemma were combined to allow the assay of Na^+/K^+-ATPase activity. This activity and quantitation of the immunoblots were also performed (see MATERIALS AND METHODS). *$P < 0.05$ compared with sarcolemma.

Kidney epithelial cells are known to be rich sources of Na^+/K^+-ATPase. It was of interest, therefore, to estimate the fraction of the total cellular content of Na^+/K^+-ATPase that is localized in the caveolar fractions of such cells. In experiments similar to those of Fig. 4, fractionations of four different samples of HEK-293 cells were done, and distribution of the α_1-subunit in various fractions was quantitated from blots and protein contents of the fractions also as described for experiments of Fig. 4. The light *fractions 4* and *5* contained 55.9 ± 3.3% (means ± SE; $n = 4$) of the total cellular content of the subunit. Comparison of this with the significantly lower value found in cardiac preparations (Fig. 4) indicates cell-specific differences in caveolar contents of Na^+/K^+-ATPase and suggests that the abundance of a membrane protein may be a significant factor in its relative distribution between the caveolae/raft microdomains, the bulk plasma membrane, and the internal membranes (27).

Ouabain effects on cardiac caveolar pools of ERK1/2, Src, and Na^+/K^+-ATPase. To begin the assessment of the role of the caveolar pool of Na^+/K^+-ATPase in the signal-transducing function of the enzyme, we focused our initial studies on the cardiac enzyme. We had shown recently (26) that ouabain-induced increase in the contractility of the intact heart is accompanied by the activation of the proximal signaling events leading to ERK1/2 activation. Therefore, we exposed the isolated rat hearts to an ouabain dose that produces positive inotropy but no toxicity, froze the control and the exposed hearts at the peak of the ouabain effect on contractility, prepared the caveolae from ventricular samples, and determined the caveolar contents of ERK1/2. The results (Fig. 9) showed that ouabain did not change the caveolar contents of ERK1/2 proteins but that the caveolar contents of the phosphorylated ERK1/2 were significantly increased by ouabain. It has been previously demonstrated (13, 15) that such an increase in the ratio of phosphorylated ERK1/2 to total ERK1/2 is indicative of ERK1/2 activation. We also compared the caveolar contents of caveolin-3, Src, and α_1- and α_2-subunits of Na^+/K^+-ATPase in the ouabain-treated and the control hearts. Ouabain did not change the caveolar contents of caveolin-3 and the α_1-subunit, but it caused significant increases in the caveolar contents of the α_2-subunit and Src (Fig. 10).

DISCUSSION

A major new finding reported here is the demonstration of the presence of a significant pool of Na^+/K^+-ATPase in the caveolae microdomains of the plasma

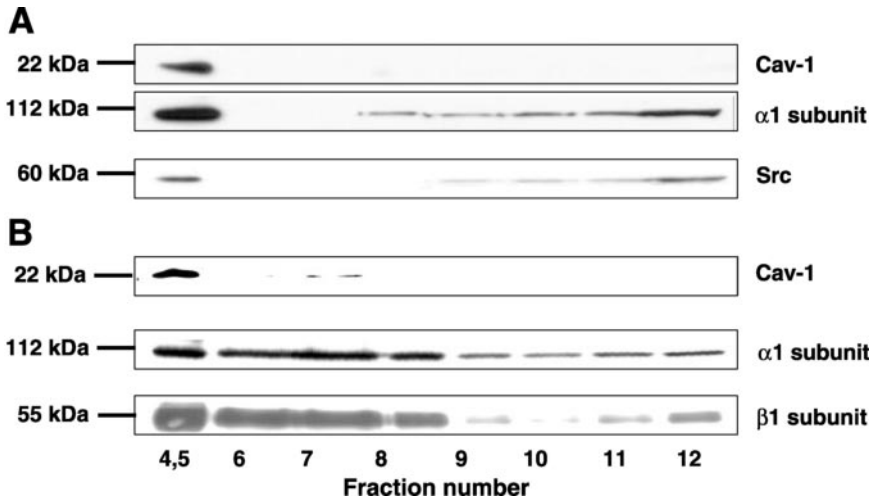

Fig. 8. Localization of Na^+/K^+-ATPase subunits in Cav-1-rich domains of human embryonic kidney (HEK)-293 cells (*A*) and pig kidney outer medulla (*B*). Sample fractionations were done as indicated in Fig. 1. Total protein distribution patterns (not shown) were similar to those shown in Fig. 1. For the immunoblots of the HEK-293 cell lysate, an equal volume from each fraction was used; for the pig kidney lysate, an equal amount of protein from each fraction was blotted.

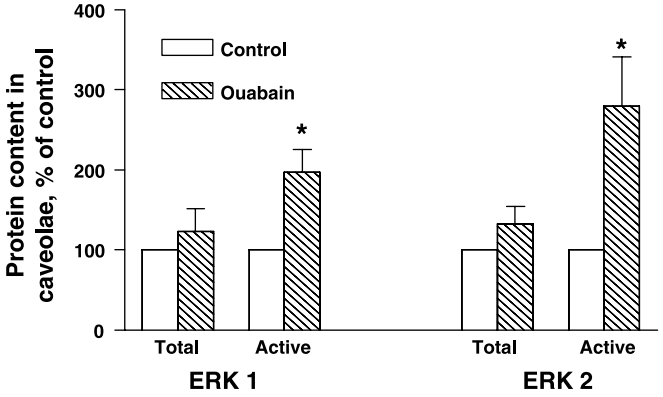

Fig. 9. Effects of ouabain-induced positive inotropy on the cardiac caveolar contents of phosphorylated ERK1/2 and total ERK1/2. Isolated rat hearts were exposed to 50 μM ouabain for 10 min to induce maximal increase in contractility (see MATERIALS AND METHODS). Ventricular samples of control and treated hearts (8 pairs) were fractionated as in Fig. 1, and caveolae (*fractions 4* and *5*) were quantitated for total immunoreactive ERK1/2 protein and immunoreactive phosphorylated ERK1/2 (active ERK1/2) as indicated in MATERIALS AND METHODS. $*P < 0.05$ compared with control.

membrane of several different cell types. It is appropriate, therefore, that at the outset we address the apparent discrepancy between this finding and those of previous reports. In several studies where caveolae were reported not to contain Na^+/K^+-ATPase (9, 18, 33), the caveolar fractions were prepared in the presence of Triton X-100. Our experiments (Fig. 6) show that Na^+/K^+-ATPase, like numerous other caveolar proteins (1, 36, 37), is indeed solubilized by Triton X-100 and removed from caveolin-containing fractions. In two other studies (5, 48), caveolae that were prepared from cardiac myocytes by the same detergent-free and carbonate-based procedure that we have used here were reported not to contain Na^+/K^+-ATPase. Detection of the enzyme in one of these studies (5) was attempted through the assay of [^{3}H]ouabain binding. Because rat cardiac myocytes were used, and the predominant Na^+/K^+-ATPase isoform of these cells (α_1) is known to be relatively insensitive to ouabain (its K_d being ~10 μM; Refs. 20, 46), it is not surprising that the caveolar presence of the enzyme was missed. In the other study (48), also on rat cardiac myocytes, we suspect that Na^+/K^+-ATPase was not detected in caveolae because of the inadequate sensitivity of the particular Western blot analysis used.

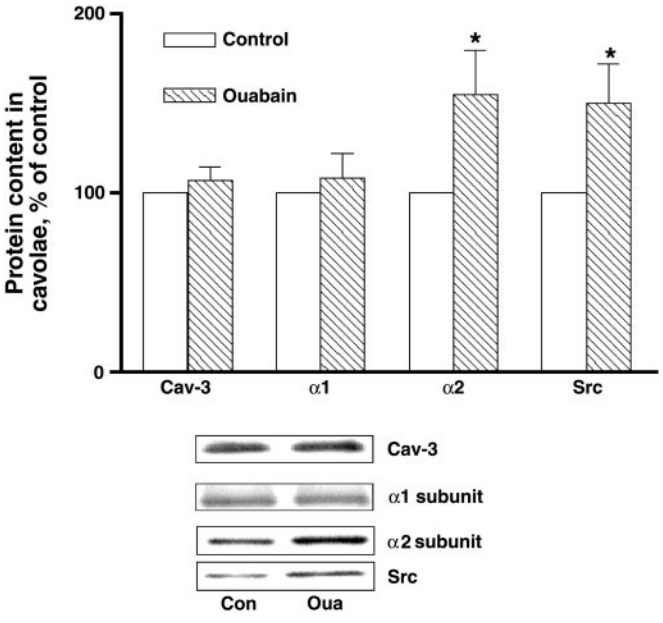

Fig. 10. Effects of ouabain-induced positive inotropy on the cardiac caveolar contents of Cav-3, Src, and the α_1- and α_2-isoforms of Na^+/K^+-ATPase. Experiments were performed as indicated in Fig. 9 and MATERIALS AND METHODS. Representative blots are shown at *bottom*, and quantitation of such blots from experiments on multiple hearts is shown at *top*. The number of pairs of control (Con) and ouabain (Oua)-treated caveolar preparations immunoassayed for the indicated proteins were as follows: Cav-3, $n = 4$; α_1-subunit, $n = 9$; α_2-subunit, $n = 13$; and Src, $n = 9$. $*P < 0.05$ compared with control.

Cardiac caveolar Na^+/K^+-ATPase and signal transduction. To explore the role of caveolar Na^+/K^+-ATPase in signal transduction, we limited our initial studies to cardiac Na^+/K^+-ATPase because most of our previous work on ouabain-induced signaling had been done on cardiac myocytes and the intact heart (26, 45). Because it is now evident that the rapid proximal signal pathways emanating from cardiac Na^+/K^+-ATPase regulate the effect of ouabain on cardiac contractility (26, 41), we sought evidence for the occurrence of these proximal pathways in the caveolae of the isolated Langendorff heart where the unambiguous positive inotropic effect of ouabain may be demonstrated.

Our previous studies on cultured cardiac myocytes and isolated heart preparations have identified the proximal pathways that link Na^+/K^+-ATPase to ERK1/2, as depicted in Fig. 11, and have established that activation of these pathways accompanies

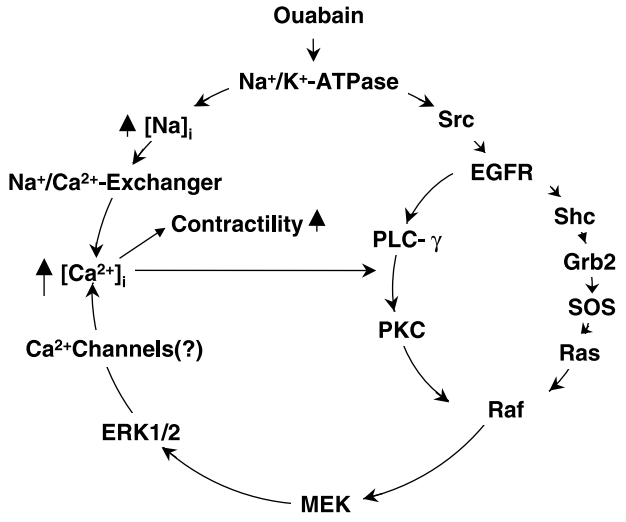

Fig. 11. The postulated integration of the signal-transducing and ion-pumping functions of Na^+/K^+-ATPase and the control of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by these functions within cardiac caveolae microdomains (see DISCUSSION). Grb2, growth factor receptor-bound protein-2; SOS, mammalian homologue of son-of-sevenless.

ouabain-induced positive inotropy (26). Significantly, our studies have also shown the existence of a positive feedback mechanism within this pathway: the requirement of the ouabain-induced rise in $[Ca^{2+}]_i$ for ERK1/2 activation (13, 25), and the necessity of ouabain-induced ERK1/2 activation for the rise in $[Ca^{2+}]_i$ (41). Because a ouabain-induced rise in $[Ca^{2+}]_i$ is known to be due to the cooperation of the pumping function of Na^+/K^+-ATPase and the transport functions of the neighboring Na^+/Ca^{2+} exchanger (22, 23, 41) and the voltage-regulated Ca^{2+} channels (21, 41), we must conclude that the signal-transducing function of cardiac Na^+/K^+-ATPase, its ion pumping function, and the effects of these on the regulation of $[Ca^{2+}]_i$ are tightly coupled within the cycle shown in Fig. 11. It is intuitively obvious that colocalization of the multiple protein components of this cycle within a restricted microdomain would be more conducive to rapid and specific stimulus-induced activation of the cycle than the random dispersion of the component proteins within the plasma membrane. Of the indicated proteins of Fig. 11, localization of the following in the caveolae of cardiac myocytes has been indicated by previous studies: PKC, Raf, MEK, and ERK1/2 (31, 32); Ras, growth factor rececptor-bound protein (4); Na^+/Ca^{2+} exchanger (2); and Ca^{2+} channels (19). Our data (Figs. 1, 2, and 5) add the subunits of cardiac Na^+/K^+-ATPase, Src, and EGFR to this list and confirm the previous findings on the caveolar presence of ERK1/2. More importantly, our data show that the constant caveolar pool of ERK1/2 is indeed activated in response to ouabain (Fig. 9), clearly indicating that concomitant with the development of the positive inotropy in the intact heart, the activation of the entire cycle of Fig. 11 may occur within the caveolae.

Stimulus-induced movements of several receptors into or out of caveolae have been demonstrated both in cardiac myocytes and other cell types (5, 14, 24), and in some cases such traffic has been shown to be relevant to signaling by the receptor. Our data show no effect of ouabain on the caveolar traffic of the predominant housekeeping isoform of Na^+/K^+-ATPase (α_1) but clearly indicate the ouabain-induced recruitments of Src and the α_2-isoform of Na^+/K^+-ATPase to caveolae (Fig. 10). This preferential ouabain-induced movement of the less abundant α_2-isoform is of particular interest in view of the previous suggestions on the possibility of a special role of the minor isoforms in the manifestation of the classic effects of ouabain on cardiac contractility. Because the positive inotropic effect of ouabain involves the cooperative functions of Na^+/K^+-ATPase and Na^+/Ca^{2+} exchanger, and because several studies have indicated the preferential concentration of the exchanger in the T tubules of the adult myocyte (23, 47), the possibility was considered (23) that a similar localization of the α_2-isoform, but not that of the α_1-isoform, in T tubules would make the minor isoform the preferred partner for the Na^+/Ca^{2+} exchanger in the regulation of $[Na^+]_i$ and $[Ca^{2+}]_i$. The findings of immunocytochemical studies, however, did not support this attractive hypothesis, showing the uniform distribution of the α_2-isoform in T tubules and the peripheral plasma membrane of the adult cardiac myocytes (23). On the other hand, similar studies on cultured cells other than cardiac myocytes (12) have indicated the preferential localization of the minor isoforms (α_2 and α_3) of Na^+/K^+-ATPase in areas of plasma membrane close to endoplasmic reticuli/sarcoplasmic reticuli (ER/SR), leading to the suggestion that ouabain effects on $[Ca^{2+}]_i$ in these cells may be through the inhibition of the minor isoforms that communicate with ER/SR within a restricted space. Evidence for a specific role of the α_2-isoform in the regulation of cardiac contractility has also been obtained from studies on cardiac function of transgenic mice with altered levels of the cardiac Na^+/K^+-ATPase isoforms (10), and the localization of the α_2-isoform in a restricted space of cardiac myocyte plasma membrane has been postulated despite of the lack of evidence for the preferential concentration of this isoform in T tubules. Perhaps in adult cardiac myocytes the fraction of the caveolae that is located on the T tubules is indeed the restricted space from where the caveolar Na^+/K^+-ATPase communicates with the SR. If so, our findings (Fig. 10) suggest that the preferential localization of the α_2-isoform in these caveolae may be induced by ouabain or other stimuli rather than being representative of the unstimulated state. The testing of this hypothesis requires the extension of the present work using experimental approaches different from those used here. Clearly, our findings open new avenues to further studies on the differential roles of the Na^+/K^+-ATPase isoforms in the control of cardiac function.

The ouabain-induced recruitment of excess Src to caveolae (Fig. 10), along with that of the α_2-isoform, is not surprising because Src seems to be the closest partner of the ouabain-stimulated Na^+/K^+-ATPase (7, 8). The evidence that Src and other Src family kinases are normal residents of caveolae in cells other than cardiac myocytes was established long ago (1, 30, 35). There is evidence to suggest that caveolar Src is inhibited by its interaction with caveolins (17), but it is also known that activated caveolar Src participates in signaling by some caveolar receptors (15, 24). To our knowledge, however, there is no prior evidence for stimulus-induced recruitment of Src to caveolae similar to that noted here. Though ouabain-induced activation of Src is essential to signaling by Na^+/K^+-ATPase (7, 8), it is not evident why the resting level of caveolar Src is not sufficient for interaction with caveolar Na^+/K^+-ATPase and why an excess of Src is recruited. Clarification of the mechanism of interaction of Src with caveolar Na^+/K^+-ATPase isoforms also requires further study.

The caveolar pool of Na^+/K^+-ATPase that is postulated to participate in the ouabain-induced events of Fig. 11 should have Na^+/K^+-ATPase activity and the associated pumping function in the absence of ouabain. This is supported by the limited but important data of Fig. 7 showing that the cardiac caveolar pool indeed exhibits ouabain-sensitive Na^+/K^+-ATPase activity. This pool, however, also has a lower specific activity

and a higher α-subunit content than the total plasma membrane pool (Fig. 7). Although the cause of this may prove to be trivial, an intriguing possibility is that the different pools within the bulk plasma membrane and the caveolae microdomains may have different ATPase and transport properties. This raises the question of whether there is independent evidence for the functional heterogeneity of Na^+/K^+-ATPase. In fact, even in the highly purified preparations of the membrane-bound Na^+/K^+-ATPase that have been used extensively for studies on the reaction mechanism, there is ample evidence for such heterogeneity and some support for lipid-phase heterogeneity being the cause of this (29). Because cholesterol has been shown to have a biphasic effect (activating followed by inhibitory) on Na^+/K^+-ATPase activity (49), and because caveolae are known to have significantly higher cholesterol content than the bulk plasma membrane (1, 35), it is reasonable to suspect a role of cholesterol, and/or caveolin-3, in the regulation of the pumping function of cardiac caveolar Na^+/K^+-ATPase. Studies on the possibility of detecting the transport function of this pool and on the potential differences between the hydrolytic and the transport functions of the caveolar and the noncaveolar pools of cardiac Na^+/K^+-ATPase are in progress.

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