Ouabain Assembles Signaling Cascades through the Caveolar Na\textsuperscript{+}/K\textsuperscript{+}-ATPase*  

Received for publication, December 4, 2003, and in revised form, February 9, 2004  
Published, JBC Papers in Press, February 12, 2004, DOI 10.1074/jbc.M31329200

Haojie Wang‡, Michael Haas‡, Man Liang‡, Ting Cai‡, Jiang Tian‡, Shengwen Li‡, and Zijian Xie‡¶  
From the ‡Departments of Pharmacology and Medicine, Medical College of Ohio, Toledo, Ohio 43614 and §Allergan, Inc., Pharmaceuticals R&D, Irvine, California 92623-9534

Based on the observation that the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase a subunit contains two conserved caveolin-binding motifs, we hypothesized that clustering of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and its partners in caveolae facilitates ouabain-activated signal transduction. Glutathione S-transferase pull-down assay showed that the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase bound to the N terminus of caveolin-1. Significantly, ouabain regulated the interaction in a time- and dose-dependent manner and stimulated tyrosine phosphorylation of caveolin-1 in LLC-PK1 cells. When added to the isolated membrane fractions, ouabain increased tyrosine phosphorylation of proteins from the isolated caveolae but not other membrane fractions. Consistently, ouabain induced the formation of a Na\textsuperscript{+}/K\textsuperscript{+}-ATPase-src-caveolin complex in the isolated caveolae preparations as it did in live cells. Finally, depletion of either cholesterol by methyl β-cyclodextrin or caveolin-1 by siRNA significantly reduced the caveolar Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and Src. Concomitantly, cholesterol depletion abolished ouabain-induced recruitment of Src to the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase signaling complex. Like depletion of caveolin-1, it also blocked the effect of ouabain on ERKs, which was restored after cholesterol repletion. Clearly, the caveolar Na\textsuperscript{+}/K\textsuperscript{+}-ATPase represents the signaling pool of the pump that interacts with Src and transmits the ouabain signals.

Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, or the sodium pump, is a ubiquitous transmembrane pump that transports Na\textsuperscript{+} and K\textsuperscript{+} across the plasma membrane by hydrolyzing ATP (1–3). The pump also functions as a signal-transducing receptor for ouabain and other cardiotoxic steroids (4). Binding of ouabain to the pump activates multiple signal transduction pathways and regulates transcription and translation of many genes in cardiac myocytes and other cell types (5–7). Significantly, some of these ouabain effects were independent of changes in intracellular ion concentrations (8–10). More recently, we have demonstrated that Na\textsuperscript{+}/K\textsuperscript{+}-ATPase interacts with neighboring membrane proteins and organized cytosolic cascades of signaling complexes to transmit the ouabain signal to different intracellular compartments (11). Subsequently, the activated Na\textsuperscript{+}/K\textsuperscript{+}-ATPase-Src complex recruits/phosphorylates multiple proteins. This eventually results in the assembly of different signaling cascades. One of the activated cascades involves Src-mediated trans-activation of EGFR (8) and subsequent recruitment and assembly of the Src/Ras/Raf/ERKs in different cell lines (11).

Realization that Na\textsuperscript{+}/K\textsuperscript{+}-ATPase has to interact with Src, EGFR, and other proteins to transmit the ouabain signal has prompted us to propose that the signaling pump is pre-assembled with its partners in membrane microdomains such as caveolae. Caveolae were first identified as flask-shaped vesicular invagination of plasma membrane and are enriched in cholesterol, glycosphingolipids, and sphingomyelin (12, 13). Caveolins are structural proteins of caveolae. The mammalian cells express three different genes (namely caveolin-1, -2, and -3) that encode five different isoforms. The primary sequence of caveolin-1 contains a central hydrophobic domain (residues 102–134) that anchors to membranes, an oligomerization domain (residues 61–101), and a scaffolding domain (residues 82–101) (13). Interaction between the oligomerization domains and the C-terminal domains results in formation of high molecular oligomers containing about 14–16 caveolins, which is important for the scaffolding function of caveolins. Interaction of caveolin scaffolding domain with putative caveolin-binding motifs in a large number of signaling proteins such as Src, EGFR, and Ras concentrates these proteins in caveolae (13). This makes caveolins an ideal microdomain for the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase to interact with Src and other signaling proteins. However, early studies failed to demonstrate that caveolae contain Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (14, 15). Because these studies used Triton X-100 to prepare caveolae, we have recently re-examined this issue using a well-established detergent-free method, because it is well established that Na\textsuperscript{+}/K\textsuperscript{+}-ATPase is readily soluble in Triton X-100. The data indicate that the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase is concentrated in caveolae isolated from the heart, the kidney, and cultured cells (16). Significantly, we found that ouabain activated the caveolar ERKs in the isolated heart preparation, suggesting that the caveolar Na\textsuperscript{+}/K\textsuperscript{+}-ATPase may be involved in ouabain-mediated signal transduction (16). Accordingly, this study aims to determine whether Na\textsuperscript{+}/K\textsuperscript{+}-ATPase directly interacts with caveolin-1 and if caveolae are actually involved in formation of a signaling module for components of the ouabain-activated Na\textsuperscript{+}/K\textsuperscript{+}-ATPase signaling complex.

* This work was supported by National Institutes of Health Grants HL-36573, HL-63238, and HL-67963, awarded by the National Heart, Lung and Blood Institute, United States Public Health Service, Department of Health and Human Services, and a pre-doctoral grant, awarded by American Heart Association-Ohio Valley Affiliate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.  

†To whom correspondence should be addressed: Dept. of Pharmacology, Medical College of Ohio, 3035 Arlington Ave., Toledo, OH 43614-5804. Tel.: 419-383-4182; Fax: 419-383-2871; E-mail: zxie@mco.edu.

‡ The abbreviations used are: EGFR, epidermal growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; Mβ-CD, methyl β-cyclodextrin; PBS, phosphate-buffered saline; PKC, protein kinase C; PP2, 4-amino-5-(4-chlorophenyl)-7-(3-t-butyl)pyrazolo[3,4-d]pyrimidine; RIPA, radioimmune precipitation assay; TGF-β, transforming growth factor β; Mes, morpholineethanesulfonic acid; siRNA, small interference RNA; MBS, Mes-buffered saline.
Caveolar Na⁺/K⁺-ATPase and Signal Transduction

EXPERIMENTAL PROCEDURES

Materials—Chemicals of the highest purity were purchased from Sigma (St. Louis, MO). The antibodies used and their sources are as follows. The anti-phospho ERK monoclonal antibody, anti-caveolin-1 monoclonal antibody, anti-Src monoclonal and polyclonal antibodies, goat anti-rabbit secondary antibody, and goat anti-mouse secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Optitran nitrocellulose membranes used for Western blotting were obtained from Schleicher and Schuell (Keene, NH). Anti-caveolin monoclonal and polyclonal antibodies were from BD Transduction Laboratories (Lexington, KY). The monoclonal anti-Src (clone GD11), polyclonal anti-αv antibody, and Protein G-agarose were obtained from Upstate Biotechnology (Lake Placid, NY). All secondary antibodies were conjugated to horseradish peroxidase; therefore, the immunoreactive bands were developed using chemiluminescence kit (Pierce, Rockford, IL).

Cell Preparation and Culture—Pig LLC-PK1 cells, human 293 cells, and SYF and SYF + Src cells were obtained from American Type Culture Collection and cultured in DMEM containing 10% fetal bovine serum, and penicillin (100 units/ml)/streptomycin (100 μg/ml). When cell cultures reached about 80% confluence, cells were serum-starved for 24 h and used for the experiments.

Measurement of ERK Activity—Immunoblotting was performed to identify the activation of ERK using the antibodies described under “Experimental Procedures” (8). Dilutions of these antibodies were done as recommended by the manufacturer. Following the indicated treatment, the incubation medium was rapidly replaced with 5 ml of iced cold PBS. The washed cells were then lysed in 200 μl of iced cold RIPA buffer containing 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM Tris-HCl (pH 7.4). Cell lysates were centrifuged at 16,000 x g for 15 min, and supernatants were used for Western blot analysis. Samples were separated by SDS-PAGE (8 μl/lane) and transferred to an Optitran membrane as we previously described (8). The membranes were then probed with an anti-phospho-ERK monoclonal antibody. The anti-phospho-ERK monoclonal antibody was then stripped, and the membrane was re-probed with a polyclonal antibody that recognizes the total amount of ERK to account for equal loading as we previously reported (8). Autoradiograms were scanned with a Bio-Rad densitometer to quantify ERK signals as previously described (8).

Purification of Caveolin-rich Membrane Fractions—Caveolin-rich membrane fractions were obtained according to the method of Song et al. (17). LLC-PK1 cells were washed with ice-cold PBS, scraped in 2 ml of 500 mM sodium carbonate, pH 11.0, and collected to a 50-ml Falcon tube. The homogenate was then adjusted to 45% sucrose by addition of 2 ml of ice-cold RIPA buffer (300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM Tris-HCl (pH 7.4)). Cell lysates were centrifuged at 16,000 x g for 15 min, and supernatants were used for Western blot analysis. Samples were separated by SDS-PAGE (8 μl/lane) and transferred to an Optitran membrane as we previously described (8). The membranes were then probed with an anti-phospho-ERK monoclonal antibody. The anti-phospho-ERK monoclonal antibody was then stripped, and the membrane was re-probed with a polyclonal antibody that recognizes the total amount of ERK to account for equal loading as we previously reported (8). Autoradiograms were scanned with a Bio-Rad densitometer to quantify ERK signals as previously described (8).

Assay for Caveolin-1 and Src Association—Caveolin-rich membrane fractions obtained according to the method of Song et al. (17). LLC-PK1 cells were washed with ice-cold PBS, scraped in 2 ml of 500 mM sodium carbonate, pH 11.0, and collected to a 50-ml Falcon tube. The homogenate was then adjusted to 45% sucrose by addition of 2 ml of ice-cold RIPA buffer (300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM Tris-HCl (pH 7.4)). Cell lysates were centrifuged at 16,000 x g for 15 min, and supernatants were used for Western blot analysis. Samples were separated by SDS-PAGE (8 μl/lane) and transferred to an Optitran membrane as we previously described (8). The membranes were then probed with an anti-phospho-ERK monoclonal antibody. The anti-phospho-ERK monoclonal antibody was then stripped, and the membrane was re-probed with a polyclonal antibody that recognizes the total amount of ERK to account for equal loading as we previously reported (8). Autoradiograms were scanned with a Bio-Rad densitometer to quantify ERK signals as previously described (8).

Assay for Tyrosine Phosphorylation in Isolated Membrane Fractions—This assay was performed according to the protocol described by Liu et al. (20). For each phosphorylation reaction, 500 μl of phosphorylation buffer (2 x minimal essential medium (pH 7.4)/160 μg/ml bovine serum albumin (fraction V)/2 mM NaF/400 μM Na₃VO₄/leupeptin (20 μg/ml)/soybean trypsin inhibitor (20 μg/ml)/2 mM MgCl₂/200 μM ATP) was added to a 1-ml Eppendorf tube. After an aliquot of caveole membrane preparations were transferred into each tube, ouabain was added to start the reaction. The samples were then incubated in a 37°C water bath for 5 min. To terminate the reactions, the samples were placed on ice, and 100 μl of 72% trichloroacetic acid was added to each sample immediately to precipitate proteins. Samples were then dissolved in SDS-sample buffer and separated by SDS-PAGE on 10% gels. Blots were probed for tyrosine phosphorylation with a monoclonal antibody (4G10) as we previously described (8).

To immunoprecipitate caveolin-1 from isolated caveolae, caveolae were first treated as described above except that 100 μg of caveole membrane preparations were used in a total of 2-ml reaction solutions. After ouabain treatment, caveolae were collected by centrifugation at 40,000 rpm with a SW65 rotor for 30 min. The pellets were dissolved in RIPA buffer and used for immunoprecipitation with the polyclonal anti-caveolin-1 antibody as described above.

In Vitro Binding Assay—For the GST pull-down assay, the purified pig kidney Na⁺/K⁺-ATPase was solubilized in RIPA, and pre-cleaned with glutathione-agarose beads. The specific activities of the purified Na⁺/K⁺-ATPase preparations used in this work were between 1200 and 1400 μmol of Pi/mg/h. The GST-fused N terminus of caveolin-1 residues 1–101 was constructed and purified on glutathione-agarose as described previously (21). Five micrograms of purified GST-fused N terminus of caveolin-1 and different amounts of RIPA buffer-solubilized Na⁺/K⁺-ATPase were incubated at 4°C for 2 h in a total volume of 1 ml of binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P40, and 0.1% phenylmethylsulfonyl fluoride). The beads were washed five times with the binding buffer, and the bound Na⁺/K⁺-ATPase was dissolved in the SDS sample buffer. Proteins were resolved on SDS-PAGE and analyzed by Western blot analysis using the Na⁺/K⁺-ATPase-specific antibodies (22).

Cholesterol Depletion and Repletion—MβCD was dissolved in DMEM and used directly. Cholesterol depletion was carried out by incubating the cells in the presence of 10 mM MβCD for 30 min at 37°C as previously described (23–28). After the cells were washed twice with serum-free medium, they were used for the experiments. Cholesterol depletion was then attempted to be reversed by adding 200 μg of cholesterol (20 μg/ml in ethanol) to 10 ml of 10% MβCD solution followed by vortexing at 40°C as described (23, 27).

Data Analysis—Data are given as the mean ± S.E. Statistical analysis of the Student’s t test was used and accepted at p < 0.05. Each presented immunoblot is representative of the similar results of at least three separate experiments.

RESULTS

Ouabain Regulates the Interaction of Na⁺/K⁺-ATPase with Caveolin-1—We have shown previously that ouabain activates multiple signaling cascades in LLC-PK1 cells. To begin addressing the role of the caveolar Na⁺/K⁺-ATPase in ouabain-induced signal transduction, we first determined how the Na⁺/K⁺-ATPase is distributed in these cells. Caveolae were isolated...
from LLC-PK1 cells by a well-established density gradient fractionation procedure, and the distribution of proteins in each fraction is consistent with our previous findings in 293 cells (16). When the fractions were assayed by Western blot for caveolar residents, we found that about 80% of caveolin-1 was in light fractions (fractions 4 and 5) together with about 50% of the Na+/H+ATPase and 30% of Src. To see if LLC-PK1 cells express other caveolin proteins, the same blots were stripped and re-probed for caveolin-2 and -3. As expected (12, 13), we could not detect the expression of caveolin-3 in these cells. Surprisingly, in contrast to some other epithelial cells (12), we found that LLC-PK1 cells only expressed very low levels of caveolin-2 (data not shown). Accordingly, the following experiments focused on the role of caveolin-1 in ouabain signaling, with a particular emphasis on its direct interaction with the Na+/K+-ATPase.

Caveolin-1 interacts with many signaling proteins via the scaffolding domain, and this interaction plays an important role in concentrating the signaling proteins in caveolae (13). Data base search revealed that the α1 subunit of mammalian Na+/K+-ATPase contains two highly conserved caveolin-binding motifs (Fig. 1A), indicating that these domains may be important for the functions of the Na+/K+-ATPase and are most likely to mediate a direct interaction with caveolin-1. To test this hypothesis, we performed in vitro GST pull-down assay. Na+/K+-ATPase was purified from pig kidney and solubilized in RIPA buffer. Different amounts of the solubilized pump were incubated with either 5 μg of GST or GST-fused N terminus (residues 1–101) of caveolin-1 (GST-N-cav). The bound Na+/K+-ATPase was purified on glutathione-agarose beads and analyzed by Western blot. As shown in Fig. 1B, the Na+/K+-ATPase interacted with GST-N-cav, but not GST.

Because the Na+/K+-ATPase resides in caveolae and can interact with caveolin-1 directly, we next determined if ouabain regulates the interaction between the pump and caveolin-1. LLC-PK1 cells were exposed to 1 μM ouabain for different times, and cell lysates were immunoprecipitated with a polyclonal anti-caveolin-1 antibody. As illustrated in Fig. 2A, ouabain caused a time-dependent increase in the amount of co-precipitated α1. The effects were rapid, and the maximal stimulation was observed after 2–5 min of ouabain exposure (Fig. 2B). Consistent with these findings, ouabain also increased co-precipitated caveolin-1 in a time-dependent manner when cell lysates were immunoprecipitated with anti-α1 anti-

![Fig. 1. Na+/K+-ATPase interacts directly with the N terminus of caveolin-1 in vitro. A, localization of two potential caveolin-binding motifs in the α1 subunit of Na+/K+-ATPase. Data base search shows that there are two highly conserved caveolin binding motifs: ΦXXXΦΦ at the N terminus and ΦXXXΦΦΦ at the C terminus. Note that Φ represents an aromatic amino acid residue, and X stands for any amino acid residue. N, M, and C stand for the N terminus, transmembrane domain, and C terminus, respectively. B, purified Na+/K+-ATPase was first solubilized in RIPA buffer, and used in GST pull-down assay as described under “Experimental Procedures.” A representative Coomassie Blue-stained gel (a) and the corresponding Western blot (b) of three independent experiments are shown. Purified Na+/K+-ATPase (10 μg) was resolved on SDS-PAGE and stained by Coomassie Blue (c). PKE, purified pig kidney Na+/K+-ATPase; α1, the α1 subunit of Na+/K+-ATPase; GST-N-cav, GST-N terminus of caveolin-1 (residues 1–101).](image-url)
body (Fig. 2C). When the dose-dependent effects of ouabain were determined in Fig. 2 (D and E), we found that 25 nM ouabain was sufficient to stimulate the association of caveolin-1 to the Na\(^+/\)K\(^+\)-ATPase in these cells. At this concentration, less than 15% of Na\(^+/\)K\(^+\)/H\(^+\)-ATPase is inhibited by ouabain (11). Although our previous work showed that the whole Na\(^+/\)K\(^+\)/H\(^+\)-ATPase (i.e. both \(\alpha_1\) and caveolin-1. The same experiments were repeated four times and two representative Western blots are shown in A and C. Panels D and E examine the dose-dependent effects of ouabain on the \(\alpha_1\) binding to caveolin-1. Panel F examines the effect of ouabain on the \(\beta_1\) binding to caveolin-1. A representative Western blot of four independent experiments is shown in D and F. Panels B and E show the quantitative data. Values are mean ± S.E. of four independent experiments and are expressed relative to a control value of 1. *, \(p < 0.05\); **, \(p < 0.01\) versus control. \(\alpha_1\), \(\alpha_1\) subunit of Na\(^+/\)K\(^+\)-ATPase; Oua, ouabain; IP, immunoprecipitation; Cav-1, caveolin-1; \(\beta_1\), \(\beta_1\) subunit of Na\(^+/\)K\(^+\)-ATPase.

**Fig. 2.** Ouabain regulates the interaction between Na\(^+/\)K\(^+\)-ATPase and caveolin-1 in a time- and dose-dependent manner. LLC-PK1 cells were treated with 1 \(\mu\)M ouabain for different times as indicated, and the cell lysates were immunoprecipitated with polyclonal anti-caveolin-1 antibody (A and B) or polyclonal anti-Na\(^+/\)K\(^+\)-ATPase \(\alpha_1\) subunit antibody (C). Immunoprecipitated complex was analyzed by Western blot for both \(\alpha_1\) and caveolin-1. The same experiments were repeated four times and two representative Western blots are shown in A and C. Panels D and E examine the dose-dependent effects of ouabain on the \(\alpha_1\) binding to caveolin-1. Panel F examines the effect of ouabain on the \(\beta_1\) binding to caveolin-1. A representative Western blot of four independent experiments is shown in D and F. Panels B and E show the quantitative data. Values are mean ± S.E. of four independent experiments and are expressed relative to a control value of 1. *, \(p < 0.05\); **, \(p < 0.01\) versus control. \(\alpha_1\), \(\alpha_1\) subunit of Na\(^+/\)K\(^+\)-ATPase; Oua, ouabain; IP, immunoprecipitation; Cav-1, caveolin-1; \(\beta_1\), \(\beta_1\) subunit of Na\(^+/\)K\(^+\)-ATPase.
that ouabain can stimulate the formation of an active Na\textsuperscript{+}/K\textsuperscript{+}-ATPase-Src-caveolin-1 module in the caveolae. To further confirm that the signaling module of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase-Src-caveolin-1 is formed in caveolae, but not other membrane fractions, we isolated caveolae from either control or ouabain-treated LLC-PK1 cells. The isolated caveolae were then solubilized in RIPA buffer and immunoprecipitated using anti-caveolin-1 antibody as in Fig. 3D. In comparison to caveolae isolated from control cells, there was a significant increase in the formation of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase-Src-caveolin-1 complex in the caveolae prepared from ouabain-treated cells (Fig. 3D).
Because ouabain stimulates tyrosine phosphorylation of multiple proteins in various cell lines, including LLC-PK1 cells, the above findings led us to propose that binding of ouabain to the caveolar Na\(^{+}/K^{+}\)-ATPase is sufficient to stimulate tyrosine phosphorylation of multiple proteins other than caveolin-1 within the caveole structure. Indeed, as illustrated in Fig. 4B, ouabain caused a dose-dependent increase in tyrosine phosphorylation of several proteins in the isolated caveola as it did in live cells. Consistently, when caveole lysates were immunoprecipitated with anti-caveolin-1 antibody, we observed that ouabain stimulated the formation of the signaling complex consisting of Na\(^{+}/K^{+}\)-ATPase, Src, and caveolin-1 as observed in LLC-PK1 cells (Fig. 4D and Ref. 10).

As shown in Fig. 4A, membrane fractions 8 and 9 also contain a significant amount of Na\(^{+}/K^{+}\)-ATPase and Src. To demonstrate that the Na\(^{+}/K^{+}\)-ATPase in caveola, but not in other membrane fractions, responds to ouabain stimulation, we repeated the experiments depicted in Fig. 4A with membranes collected from fractions 8 and 9. The data showed that, although multiple proteins from these membrane fractions were tyrosine-phosphorylated, ouabain had no effect on protein tyrosine phosphorylation (Fig. 4C). These findings are consistent with the notion that the Na\(^{+}/K^{+}\)-ATPase interacts with caveolin-1 (Figs. 4A and Ref. 10).

To test if the protein tyrosine phosphorylation is mediated by ouabain-induced activation of Src, the isolated caveole were first treated with Src inhibitor PP2, and then exposed to ouabain. Western blot analysis showed that PP2 abolished ouabain-induced tyrosine phosphorylation (Fig. 5A). To further confirm the role of Src, we repeated these experiments in the isolated caveola from SYF and SYF+c-Src cells. As depicted in Fig. 5B, although ouabain stimulated tyrosine phosphorylation in caveola isolated from SYF+c-Src cells, it failed to do so in the caveola isolated from SYF cells.

Depletion of Either Cholesterol or Caveolin-1 Moves Na\(^{+}/K^{+}\)-ATPase and Src Out of Caveola—So far, we have demonstrated that the caveolar Na\(^{+}/K^{+}\)-ATPase interacts with caveolin-1 and that ouabain activates the caveolar Na\(^{+}/K^{+}\)-ATPase to transmit its signals to LLC-PK1 cells. To further test the functional role of caveola in ouabain-activated signal transduction, LLC-PK1 cells were treated with 10 mM MJ-CD for different times to deplete cholesterol from the plasma membrane. Because depletion of cholesterol has been shown to disrupt the structure of caveola (22–28), we reasoned that MJ-CD might affect the ability of the cell to concentrate the Na\(^{+}/K^{+}\)-ATPase and its signaling partners in caveola. Indeed, Western blot analysis showed that, although MJ-CD had marginal effects on caveolin-1 content in the isolated caveola, it caused more than 50% reduction in the caveolar Na\(^{+}/K^{+}\)-ATPase and Src (Fig. 6). Because the total protein recovered in the caveola fraction was not altered by cholesterol depletion (data not shown) as previously reported (23), and MJ-CD had no effect on total cellular Na\(^{+}/K^{+}\)-ATPase and Src content (data not shown), the above findings indicate that removal of caveolar cholesterol re-distributes the Na\(^{+}/K^{+}\)-ATPase and Src in LLC-PK1 cells.

Because the Na\(^{+}/K^{+}\)-ATPase interacts with caveolin-1 (Figs. 6A and Ref. 10), the above findings indicate that removal of caveolar cholesterol re-distributes the Na\(^{+}/K^{+}\)-ATPase and Src in LLC-PK1 cells.
Na⁺/K⁺-ATPase and Signal Transduction

In this report we have made several novel and important observations. First, we demonstrated that the signaling Na⁺/K⁺-ATPase interacts with caveolin-1 in a ligand-dependent manner and that the interaction is important for the signaling...
pump to transmit the ouabain signal. Second, binding of ouabain to the caveolar Na\(^+/\)K\(^+-\)ATPase is sufficient to activate signaling cascades. Finally, the Na\(^+/\)K\(^+-\)ATPase, together with its signaling partners, is concentrated in caveolae in a cholesterol- and caveolin-1-dependent manner, and this makes it possible for ouabain to activate the signaling function of the pump. All together, these new findings provide new insight into the molecular mechanism of the Na\(^+/\)K\(^+-\)ATPase-mediated signal transduction.

Ouabain Regulates the Interaction between the Na\(^+/\)K\(^+-\)ATPase and Caveolin-1—Data base search identified two potential caveolin-binding motifs in the pig α\(_1\) subunit of Na\(^+/\)K\(^+-\)ATPase (e.g. ΦXXΦXXXΦ and ΦXXΦϕΧΧΦ), where Φ represents an aromatic amino acid residue. Whereas the N-terminal binding motif is located in the cytosolic side of the first transmembrane helix, the C-terminal binding motif is located extracellularly. Because these two binding motifs are highly conserved in mammalian Na\(^+/\)K\(^+-\)ATPase (Fig. 1A), we suggest that they must be important for the functions of Na\(^+/\)K\(^+-\)ATPase. Specifically, they may mediate the interaction of the pump with caveolin. Indeed, in vitro GST-pull-down assay showed that Na\(^+/\)K\(^+-\)ATPase bound to the scaffolding domain of caveolin-1 (Fig. 1B). However, it remains to be determined which caveolin-binding motifs are involved in this interaction. Because caveolins do not cross the plasma membrane, it is less likely that the C-terminal binding motif of the α\(_1\) contributes significantly to the interaction. However, because there is evidence that the transmembrane helices M9 and M10 can slip out of the membrane to either side (31, 32), interaction of this extracellular binding domain with caveolin-1 could promote M10 retention in the cytosol. Alternatively, because there is evidence that caveolin-1 can be secreted from cells (33–36) and may participate in interaction with matrix proteins at cell surface (37, 38), we suggest that the C-terminal binding motif may play a role under these conditions. Clearly, these possibilities need to be tested experimentally in the future.

Interestingly, unlike in other signaling proteins such as endothelial nitric-oxide synthase and EGFR (39), the caveolin-binding motifs are located at sites far away from the catalytic P and N domains of the α subunits. Thus, it is conceivable that binding to caveolin-1 shall not affect the ion pumping function of the pump while it can concentrate the pump and other signaling proteins into caveolae.

Although caveolin-1 has been found to interact with multiple proteins (12, 13, 38), only a few studies have demonstrated the ligand-regulated interactions (40–43). Interestingly, in all of the ligand-regulated interactions, binding of caveolin-1 makes a great impact (either positive or negative) on ligand-activated signal transduction. For example, the interaction of caveolin-1 with TGF-β type I receptor is induced by TGF-β to rapidly dampen the signaling process activated by the TGF-β receptor complex (40). On the other hand, the interactions of caveolin-1 with androgen receptor and estrogen receptor appear to enhance androgen- and estrogen-dependent as well as estrogen-independent signal transductions (41–43). Based on the find-

ERKs. The anti-phospho-ERK monoclonal antibody was then stripped, and the membrane was reprobed with a polyclonal antibody that recognizes the total amount of ERK. A and B, two representative blots of three independent experiments. C, quantitative data. D and E, effects of depletion of caveolin-1 on ouabain-induced ERK activation. Both control (pSuppressor) and caveolin-1-depleted cells (pSuppressor cav-1) were treated with 1 μM ouabain for different times and assayed for ERK activation as in A and B. A representative of three independent experiments is shown (D). E, quantitative data. In C and E, values are mean ± S.E. of three independent experiments and are expressed relative to a control value of 1. p-ERK, phosphorylated ERK; T-ERK, total ERK, *, p < 0.05 versus control.
ings presented in Figs. 4–6, we believe that ouabain-induced interaction of Na\(^{+}/K\(^{-}\)\)-ATPase with caveolin-1 is essential for ouabain-activated signal transduction in LLC-PK1 cells. First, because caveolin-1 interacts with Src and many other signaling proteins such as EGFR and Ras, this interaction will bring these proteins to the ouabain-activated Na\(^{+}/K\(^{-}\)\)-ATPase-Src complex to assemble different signaling modules. The findings presented in Figs. 6 and 7 are consistent with this notion. Second, although the precise role of the ouabain-induced phosphorylation of caveolin-1 Tyr-14 remains to be established, it will generate additional protein-protein interaction to add more signaling proteins to the ouabain-activated modules or participate in transportation of an active module to different cellular compartments (30, 44–46). Finally, because caveolin-1 binds and keeps Src in an inactive form via the scaffolding domain (47), ouabain-induced binding of caveolin to the Na\(^{+}/K\(^{-}\)\)-ATPase, at least in principle, could release and activate the caveolin-1-bound Src. Consistent with this notion, it was reported that interaction of integrin with extracellular matrix stimulated the formation of integrin-caveolin-1-Fyn complex, leading to the activation of Fyn, a Src family kinase (37).

**Compartmentation and Na\(^{+}/K\(^{-}\)\)-ATPase-mediated Signal Transduction**—Recently, we have demonstrated that Src is involved in transmission of the ouabain signal from the Na\(^{+}/K\(^{-}\)\)-ATPase to several downstream pathways (11). This process is initiated by ouabain-induced activation of a “binary” receptor consisting of Na\(^{+}/K\(^{-}\)\)-ATPase and Src, which in turn transactivates EGFR, resulting in tyrosine phosphorylation of Shc and activation of Ras/Raf/ERK1/2 cascade in LLC-PK1 cells. Because the Na\(^{+}/K\(^{-}\)\)-ATPase has to interact with Src, EGFR, and other proteins to transmit the ouabain signal, the finding of co-localization of the Na\(^{+}/K\(^{-}\)\)-ATPase with Src and EGFR in caveolae led us to propose that compartmentalization of these proteins into a compact micro-domain may facilitate the interactions among these proteins. The following observations strongly support this notion. First, we showed that ouabain induced the formation of the Na\(^{+}/K\(^{-}\)\)-ATPase-Src-caveolin-1 complex and increased tyrosine phosphorylation of caveolin-1 in LLC-PK1 cells. In addition, ouabain was also able to stimulate tyrosine phosphorylations of several proteins in isolated caveolae, but not other membrane fractions that contain both Na\(^{+}/K\(^{-}\)\)-ATPase and Src. Thus, the caveolar Na\(^{+}/K\(^{-}\)\)-ATPase, but not the pump in other membrane fractions, most likely behaves as a signal transducer for ouabain. Significantly, as in live LLC-PK1 cells, it is the activated Src that mediated ouabain-induced protein tyrosine phosphorylation in caveolae. This is consistent with the findings that ouabain-induced activation of the Na\(^{+}/K\(^{-}\)\)-ATPase-Src complex is essential for the initiation of the ouabain signaling cascades (11). Second, we showed that depletion of caveolin-1 by siRNA removed both Na\(^{+}/K\(^{-}\)\)-ATPase and Src out of caveolae. This is consistent with the notion that interaction of the Na\(^{+}/K\(^{-}\)\)-ATPase with caveolin-1 plays an important role in clustering the signaling pump in caveolae. Third, there is evidence that caveolar cholesterols not only plays a key role in maintaining the structural properties of caveolae but also regulates the distribution of many signaling proteins in caveolae (16). For example, reduction of caveolar cholesterol by either Mβ-CD or progestrone was found to cause migration of Src out of caveolae into the bulk plasma membrane (23, 28). We confirmed this in LLC-PK1 cells (Fig. 6). In addition, we showed that cholesterol depletion significantly reduced the caveolar Na\(^{+}/K\(^{-}\)\)-ATPase. Significantly, immunoprecipitation of cell lysates with anti-caveolin-1 antibody showed that ouabain induced the formation of a Na\(^{+}/K\(^{-}\)\)-ATPase-Src-caveolin-1 signaling complex in control but not cholesterol-depleted LLC-PK1 cells (Fig. 8A). Because Mβ-CD had no effect on cell viability, total cellular contents of Na\(^{+}/K\(^{-}\)\)-ATPase and Src (data not shown), and EGF-induced activation of ERK1/2 (Fig. 9A), these data indicated that reduction of the caveolar Na\(^{+}/K\(^{-}\)\)-ATPase and Src prevented these proteins to interact with each other in response to ouabain. Fourth, when cell lysates were immunoprecipitated with anti-α1, antibody, depletion of cholesterol also prevented the formation of a Na\(^{+}/K\(^{-}\)\)-ATPase-Src complex. This provides additional evidence that the Na\(^{+}/K\(^{-}\)\)-ATPase can function as a signal transducer only when the pump is concentrated with Src in caveolae. Finally, because activation of the Na\(^{+}/K\(^{-}\)\)-ATPase-Src complex is essential for ouabain-induced transactivation of the EGFR and subsequent stimulation of Ras/Raf/ERK1/2 cascade, the fact that ouabain failed to stimulate ERK1/2 in the caveolin-1-depleted cells or in the Mβ-CD pre-treated cells provides additional support of the above notion, which is further endorsed by the cholesterol repulsion study. Collectively, the data indicate that clustering Na\(^{+}/K\(^{-}\)\)-ATPase with its signaling partners by caveolin-1 in the caveolar microdomain is essential for the pump to interact with its signaling partners and generate signals in response to ouabain stimulation.

In short, we demonstrated in this report that the Na\(^{+}/K\(^{-}\)\)-ATPase interacts with caveolin-1. Ouabain regulates the interaction and induces the formation of the Na\(^{+}/K\(^{-}\)\)-ATPase-Src-caveolin-1 signaling complex. Compartmentation of the Na\(^{+}/K\(^{-}\)\)-ATPase with its signaling partners in caveolae is essential for ouabain-induced formation of the above complex and subsequent activation of Ras/Raf/ERK cascade. These findings are significant in several aspects. First, they illustrated how ouabain-activated Na\(^{+}/K\(^{-}\)\)-ATPase transduces signals through protein-protein interactions and how membrane microdomain makes this process possible. Second, we have previously demonstrated that the signal transducing function of Na\(^{+}/K\(^{-}\)\)-ATPase is essential for ouabain-induced regulation of intracellular calcium and that ouabain activates caveolar ERKs in the heart (10, 16). Because there is evidence that most of calcium influx take place in caveolae (48, 49), the new findings bring about an important question as to whether ouabain regulates intracellular calcium (10) and contractility in cardiac myocytes through caveolar Na\(^{+}/K\(^{-}\)\)-ATPase-mediated signal transduction (50). In addition, it will be important to test the role of caveolae in ouabain-induced calcium signaling in other types of cells. Finally, there is evidence that cholesterol regulates Na\(^{+}/K\(^{-}\)\)-ATPase activity. Because caveolin-1 binds cholesterol and enriches cholesterol in caveolae, it will be of interest to test if and how interaction of Na\(^{+}/K\(^{-}\)\)-ATPase with caveolin-1 affects the pumping function of the pump.

**Acknowledgment**—We thank Dr. S. Pierre for advice and comment on the manuscript.

**REFERENCES**

15. Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka,