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Ouabain Assembles Signaling Cascades through the Caveolar Na^+/K^+-ATPase*

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Based on the observation that the Na^+/K^+-ATPase α subunit contains two conserved caveolin-binding motifs, we hypothesized that clustering of the Na^+/K^+-ATPase and its partners in caveolae facilitates ouabain-activated signal transduction. Glutathione *S*-transferase pull-down assay showed that the Na^+/K^+-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^+/K^+-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^+/K^+-ATPase and Src. Concomitantly, cholesterol depletion abolished ouabain-induced recruitment of Src to the Na^+/K^+-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^+/K^+-ATPase represents the signaling pool of the pump that interacts with Src and transmits the ouabain signals.

Na^+/K^+-ATPase, or the sodium pump, is a ubiquitous transmembrane pump that transports Na^+ and K^+ across the plasma membrane by hydrolyzing ATP (1–3). The pump also functions as a signal-transducing receptor for ouabain and other cardiotonic 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^+/K^+-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^+/K^+-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[1] and subsequent recruitment and assembly of the Shc/Ras/Raf/ERKs in several different cell lines (11).

Realization that Na^+/K^+-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 cavolin-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 caveolae an ideal microdomain for the Na^+/K^+-ATPase to interact with Src and other signaling proteins. However, early studies failed to demonstrate that caveolae contain Na^+/K^+-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^+/K^+-ATPase is readily soluble in Triton X-100. The data indicate that the Na^+/K^+-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^+/K^+-ATPase may be involved in ouabain-mediated signal transduction (16). Accordingly, this study aims to determine whether Na^+/K^+-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^+/K^+-ATPase signaling complex.

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[1] 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-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; RIPA, radioimmune precipitation assay; TGF-β, transforming growth factor β; Mes, 4-morpholineethanesulfonic acid; siRNA, small interference RNA; MBS, Mes-buffered saline.

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-ERK polyclonal 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-α_1 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 ice-cold PBS. The washed cells were then lysed in 200 μl of ice-cold RIPA buffer containing 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 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 $\times$ *g* for 15 min, and supernatants were used for Western blot analysis. Samples were separated by SDS-PAGE (60 μg/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 reprobed 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 quantitate 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 centrifuge tube. The cell lysate was then homogenized using a Polytron tissue grinder (three 6-s bursts) and sonicated with three 40-s bursts. The homogenate was then adjusted to 45% sucrose by addition of 2 ml of 90% sucrose prepared in MBS (25 mM Mes, 0.15 M NaCl, pH 6.5) and placed at the bottom of an ultracentrifuge tube. The ultracentrifuge tubes were then loaded with 4 ml of 35% sucrose and 4 ml of 5% sucrose (both in MBS containing 250 mM sodium carbonate) and centrifuged at 39,000 rpm for 16–20 h in an SW41 rotor (Beckman Instruments). A light-scattering band at the interface between the 5 and 35% sucrose gradients was observed. Eleven gradient fractions of 1 ml were collected from the top to the bottom of the centrifuge tube. Among the 11 fractions, fractions 4 and 5 were combined and diluted with 4 ml of MBS, then centrifuged at 40,000 rpm with a Beckman type 65 rotor for 1 h. The pellets were resuspended in 250 μl of MBS and are termed as caveolae fraction. Samples of 10-μl caveolae fraction and 40 μl each of other fractions were subjected to SDS-PAGE and Western blot analysis.

Assay for Caveolin-1 and Src Association—Cells were lysed in RIPA buffer as described above. Cell lysates were cleared by centrifugation at 16, 000 $\times$ *g* for 15 min at 4 °C, and the supernatants were immunoprecipitated using a polyclonal anti-Na^+/K^+-ATPase α_1 subunit antibody or a polyclonal anti-caveolin-1 antibody as previously described (11). The immunoprecipitates were dissolved in sample buffer, separated on 10% SDS-PAGE, blotted on to membrane, and probed with the monoclonal anti-Na^+/K^+-ATPase α_1 subunit antibody and polyclonal anti-pY14 caveolin-1 antibody. To determine if caveolin-1 and Src bind to the Na^+/K^+-ATPase, the same blots were stripped and reprobed with monoclonal anti-Src antibody and monoclonal anti-caveolin-1 antibody, respectively. In the immunoprecipitation experiments with anti-Na^+/K^+-ATPase α_1 subunit antibody, caveolin-1 and Src signals are normalized against Western blot signal for α_1, whereas Western blot signals are normalized against caveolin-1 in the immunoprecipitation experiments with anti-caveolin-1 antibody.

Depletion of Caveolin-1 by siRNA—Caveolin-1-specific siRNA was designed based on the strategy developed by Elbashir *et al.* (18, 19). To construct human caveolin-1-specific siRNA expression vector, we first annealed the following two oligonucleotides: sense, TCG AGC CAG AAG GGA CAC ACA GTT TTC AAG AGA AAC TGT GTG TCC CTT CTG GTT TTT; antisense, CTA GAA AAA CCA GAA GGG ACA CAC AGT TTC TCT TGA AAA CTG TGT GTC CCT TCT GGC. The annealed insert was then cloned into pSuppressor™-U6 vector (BioCarta) that was digested with SalI and XbaI. The positive clone was confirmed by DNA sequencing. Transient transfection assay in 293 cells showed that expression of the above human caveolin-1-specific siRNA (C2) caused 50% decrease in caveolin-1 after 48 h post transfection. To generate a stable cell line, 293 cells were co-transfected with either 7 μg of C2 or empty pSuppressor (used as a control), together with 1 μg of pBabe-Puro that expresses puromycin resistant gene using 20 μl of LipofectAMINE™ 2000. After 24 h, the cells were selected with 1 μg/ml puromycin, and puromycin-resistant colonies were cloned and expanded.

Assay of 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$\times$ minimal essential medium (pH 7.4)/160 μg/ml bovine serum albumin (fraction V)/2 mM NaF/400 μM Na_3VO_4/leupeptin (20 μg/ml)/soybean trypsin inhibitor (20 μg/ml)/2 mM $MgCl_2$/200 μM ATP) was added to a 1-ml Eppendorf tube. After an aliquot of caveolae 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 caveolae 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 pallets 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 P_i/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, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100). 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—Mβ-CD was dissolved in DMEM and used directly. Cholesterol depletion was carried out by incubating the cells in the presence of 10 mM Mβ-CD 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 repletion was done as previously reported (23, 27). Briefly, 400 μl of a cholesterol/Mβ-CD stock solution was added to 10 ml of DMEM, and cholesterol-depleted cells were incubated in this medium for 1 h at 37 °C. A stock solution of cholesterol/Mβ-CD mixture was prepared by adding 200 μl of cholesterol (20 mg/ml in ethanol) to 10 ml of 10% Mβ-CD solution via vortexing at 40 °C as described (23, 27).

Analysis of Data—Data are given as the mean ± S.E. Statistical analysis was performed using the Student's *t* test, and significance was 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

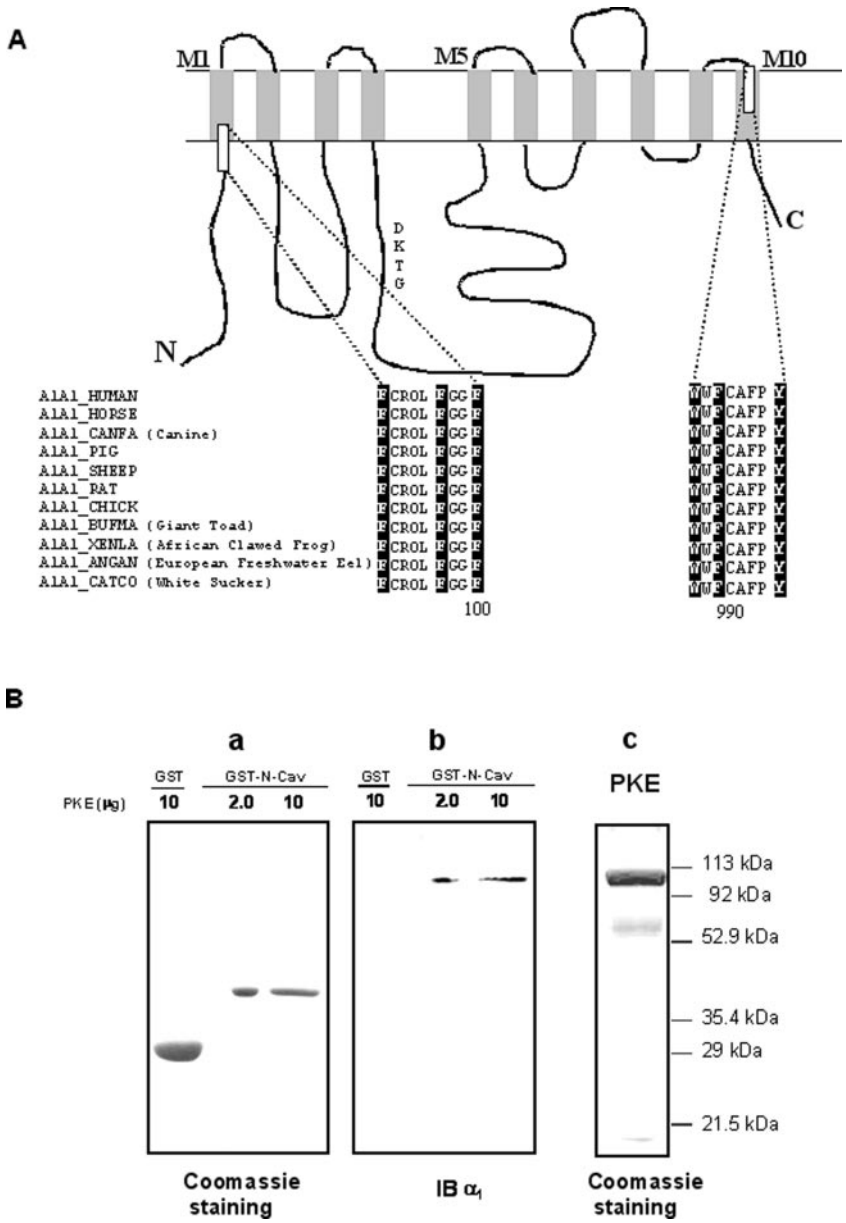

FIG. 1. **Na^+/K^+-ATPase interacts directly with the N terminus of caveolin-1 *in vitro*.** *A*, localization of two potential caveolin-binding motifs in the α_1 subunit of Na^+/K^+-ATPase. Data base search shows that there are two highly conserved caveolin binding motifs: Φ*XXXX*Φ*XX*Φ at the N terminus and Φ*X*Φ*XXXX*Φ 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; α_1, the α_1 subunit of Na^+/K^+-ATPase; *GST-N-cav*, GST-N terminus of caveolin-1 (residues 1–101).

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^+/K^+-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 α_1 subunit of mammalian Na^+/K^+-ATPase contains two highly conserved caveolin-binding motifs (Fig. 1*A*), 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. 1*B*, 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. 2*A*, ouabain caused a time-dependent increase in the amount of co-precipitated α_1. The effects were rapid, and the maximal stimulation was observed after 2–5 min of ouabain exposure (Fig. 2*B*). Consistent with these findings, ouabain also increased co-precipitated caveolin-1 in a time-dependent manner when cell lysates were immunoprecipitated with anti-a_1 anti-

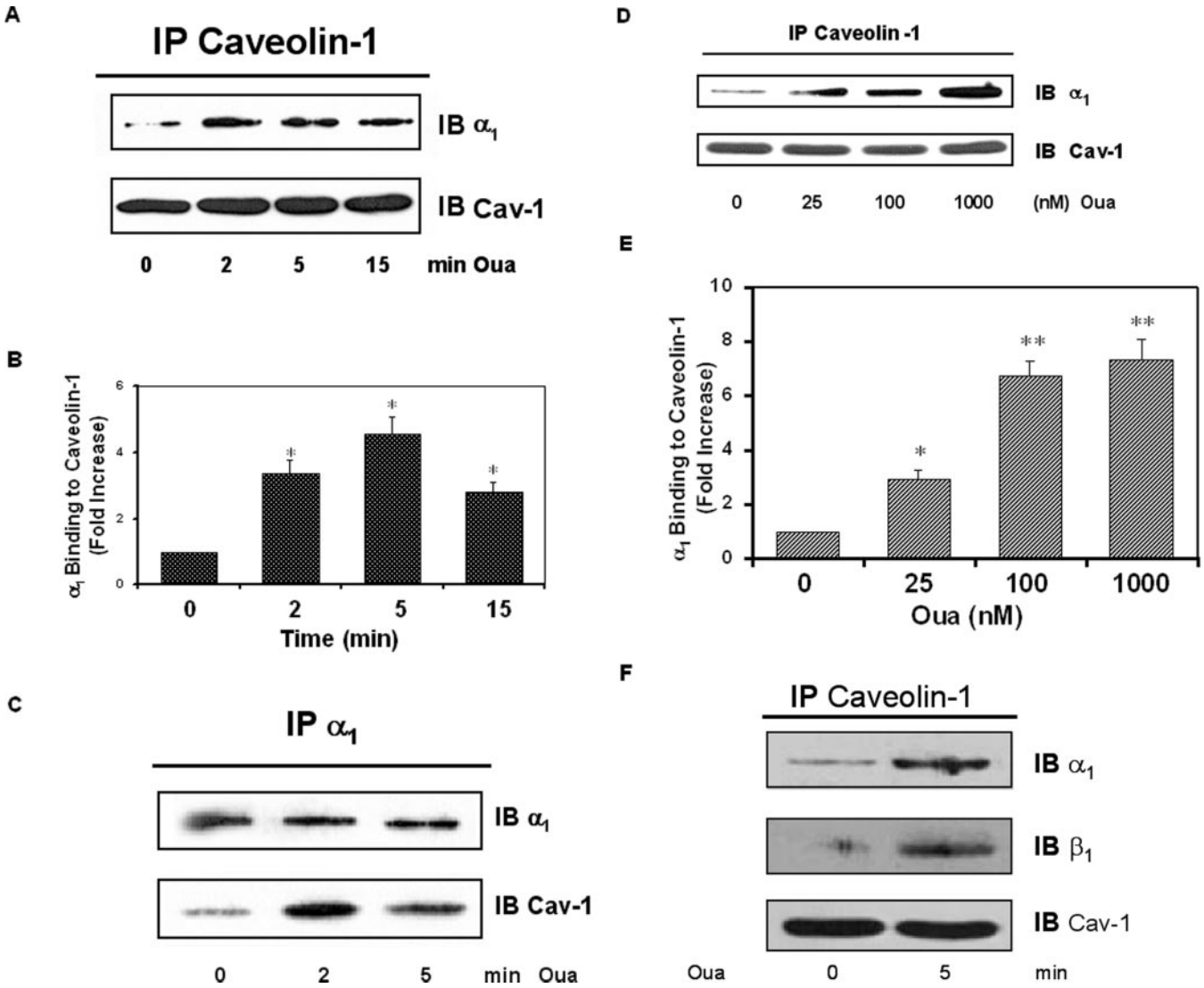

FIG. 2. **Ouabain regulates the interaction between Na^+/K^+-ATPase and caveolin-1 in a time- and dose-dependent manner.** LLC-PK1 cells were treated with 1 μM ouabain for different times as indicated, and the cell lysates were immunoprecipitated with polyclonal anti-caveolin-1 antibody (*A* and *B*) or polyclonal anti-Na^+/K^+-ATPase α_1 subunit antibody (*C*). Immunoprecipitated complex was analyzed by Western blot for both α_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 α_1 binding to caveolin-1. *Panel F* examines the effect of ouabain on the β_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. α_1, α_1 subunit of Na^+/K^+-ATPase; *Oua*, ouabain; *IP*, immunoprecipitation; *Cav-1*, caveolin-1; β_1, β_1 subunit of Na^+/K^+-ATPase.

body (Fig. 2*C*). 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^+-ATPase is inhibited by ouabain (11). Although our previous work showed that the whole Na^+/K^+-ATPase (*i.e.* both α and β subunits) is enriched in caveolae (16), to ensure that the above ouabain-regulated interaction involves the whole enzyme, we repeated the experiments of Fig. 2*A*. As shown in Fig. 2*F*, exposure of LLC-PK1 cells to 1 μM ouabain for 5 min not only increased co-precipitated α1, but also the β1 subunit. These findings clearly demonstrate that the Na^+/K^+-ATPase interacts with caveolin-1 and that ouabain regulates this interaction in LLC-PK1 cells.

Binding of Ouabain to the Caveolar Na^+/K^+-ATPase Assembles Active Signaling Modules—Because the above data indicate that the caveolar Na^+/K^+-ATPase can respond to ouabain stimulation, we tested whether binding of ouabain to the caveolar pump is able to assemble active signaling modules. We showed previously that ouabain stimulated Src in LLC-PK1 cells (11). According to prior studies (29, 30), if ouabain can activate the caveolar Na^+/K^+-ATPase-Src complex, it will stimulate tyrosine phosphorylation of caveolin-1. The first logical experiment was therefore to determine whether caveolin-1 was tyrosine-phosphorylated in response to ouabain. In the experiments illustrated in Fig. 3*A*, cells were treated with ouabain for different times and cell lysates were immunoprecipitated with a monoclonal anti-Src antibody. Western blot analysis of the immunoprecipitates clearly showed that ouabain stimulated caveolin-1 Tyr-14 phosphorylation in a time-dependent manner. These findings were confirmed when the cell lysates were immunoprecipitated with an anti-caveolin-1 antibody, then probed for Tyr-14-phosphorylated caveolin-1 (Fig. 3*B*). To ensure that Src is involved in the ouabain-induced tyrosine phosphorylation of caveolin-1, we pretreated the cells with PP2, an Src inhibitor, and then repeated the above experiments. As depicted in Fig. 3*C*, PP2 abolished ouabain-induced tyrosine phosphorylation of caveolin-1. These findings indicate

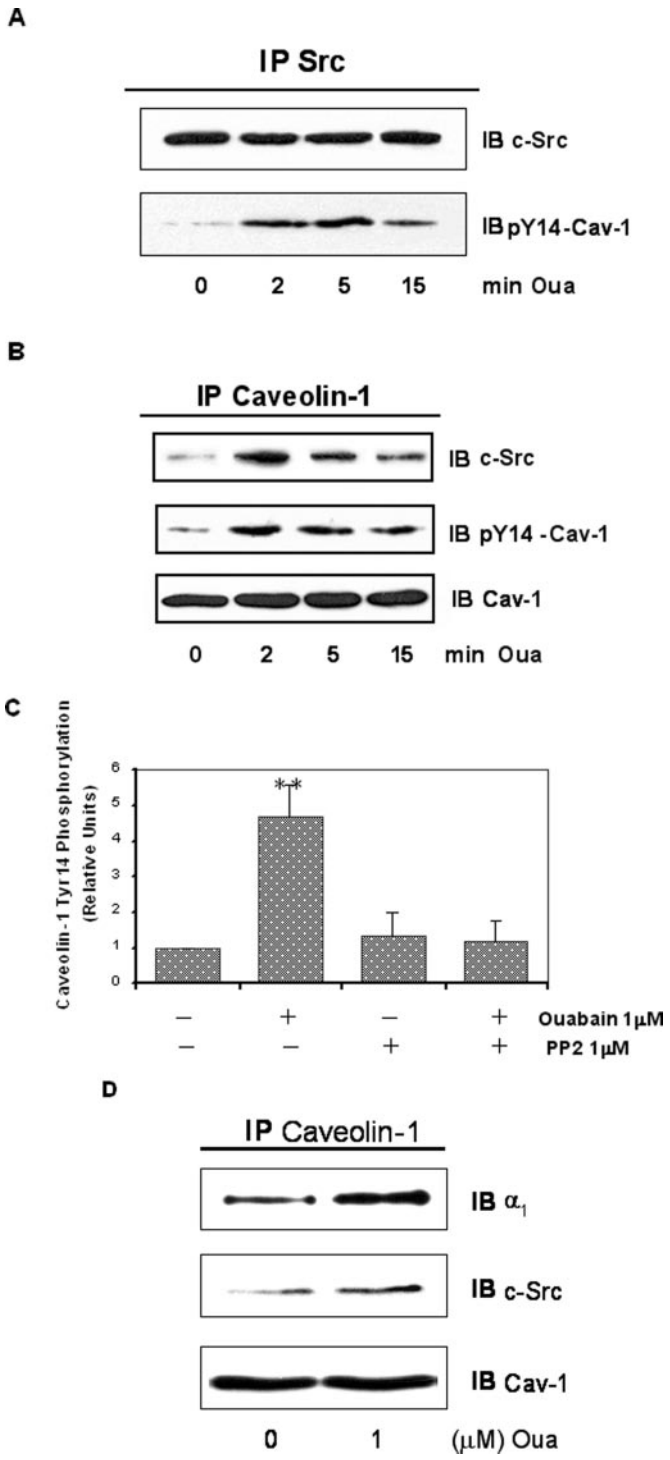

FIG. 3. **Ouabain stimulates tyrosine phosphorylation of caveolin-1 in an Src-dependent manner.** LLC-PK1 cells were treated with 1 μM ouabain for 2, 5, and 15 min, and cell lysates were immunoprecipitated with either monoclonal anti-Src antibody (*A*) or polyclonal anti-caveolin-1 antibody (*B*). The immunocomplexes were probed for Src, caveolin-1, and pY14-caveolin-1. The same experiments were repeated three times, and two representative Western blots are shown in *A* and *B*. In *C*, LLC-PK1 cells were pre-treated with 1 μM PP2 for 15 min. Both control and PP2-treated cells were then exposed to 1 μM ouabain for 5 min. Cell lysates were immunoprecipitated with anti-caveolin-1 antibody and processed as in *B*. The values are mean ± S.E. of three independent experiments. Data are expressed relative to a control value of 1. In *D*, LLC-PK1 cells were treated with or without 1 μM ouabain for 5 min. Caveolae were purified from either control or ouabain-treated cells, solubilized in RIPA buffer and immunoprecipitated with polyclonal anti-caveolin-1 antibody. A representative blot of three independent experiments is shown. α_1, α_1 subunit of Na^+/K^+-ATPase; *Cav-1*, caveolin-1; *Oua*, ouabain. **, $p < 0.01$ *versus* control.

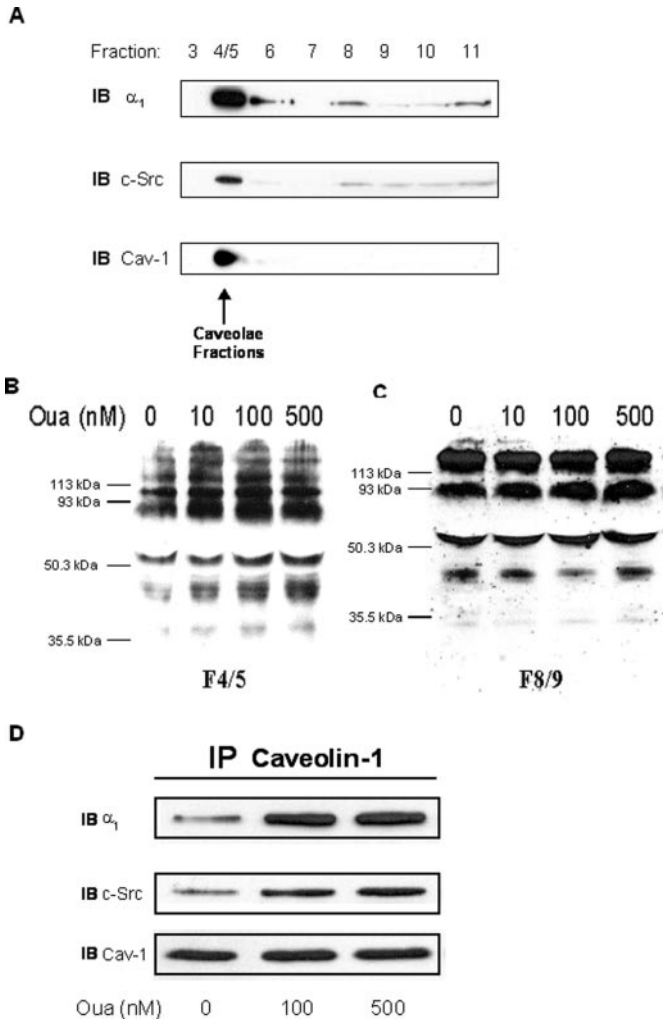

FIG. 4. **Ouabain induces tyrosine phosphorylation and formation of an active signaling complex in the isolated caveolae.** After homogenization and sonication, LLC-PK1 cell lysate was fractionated by gradient centrifugation as described under "Experimental Procedures." Fractions (1 ml each) were collected from *top* to *bottom*, assayed for total protein, and immunoblotted for proteins as indicated. *A*, a representative Western blotting showing the distribution of the indicated proteins among different fractions. *B*, aliquots of the isolated caveolae (15 μg each) were suspended in phosphorylation buffer and exposed to different concentrations of ouabain for 5 min at 37 °C. Reactions were stopped by addition of ice-cold trichloroacetic acid. Protein precipitants were dissolved in sample loading buffer, subjected to SDS-PAGE, and analyzed by Western blot with monoclonal anti-phosphotyrosine antibody. *C*, membrane proteins from fractions 8 and 9 collected during caveolae purification were combined and analyzed for ouabain-induced tyrosine phosphorylation as in *B*. *D*, aliquots of the isolated caveolae (100 μg each) were treated with indicated concentrations of ouabain as in *A*. Afterward, the samples were centrifuged at 40,000 rpm for 30 min, and the pellets were dissolved in RIPA buffer and subjected to immunoprecipitation with anti-caveolin-1 antibody. All of the above experiments were repeated at least three times. *IB*, immunoblotting; α_1, α_1 subunit of Na^+/K^+-ATPase; *Cav-1*, caveolin-1.

that ouabain can stimulate the formation of an active Na^+/K^+-ATPase-Src-caveolin-1 module in the caveolae. To further confirm that the signaling module of Na^+/K^+-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. 3*B*. In comparison to caveolae isolated from control cells, there was a significant increase in the formation of the Na^+/K^+-ATPase-Src-caveolin-1 complex in the caveolae prepared from ouabain-treated cells (Fig. 3*D*).

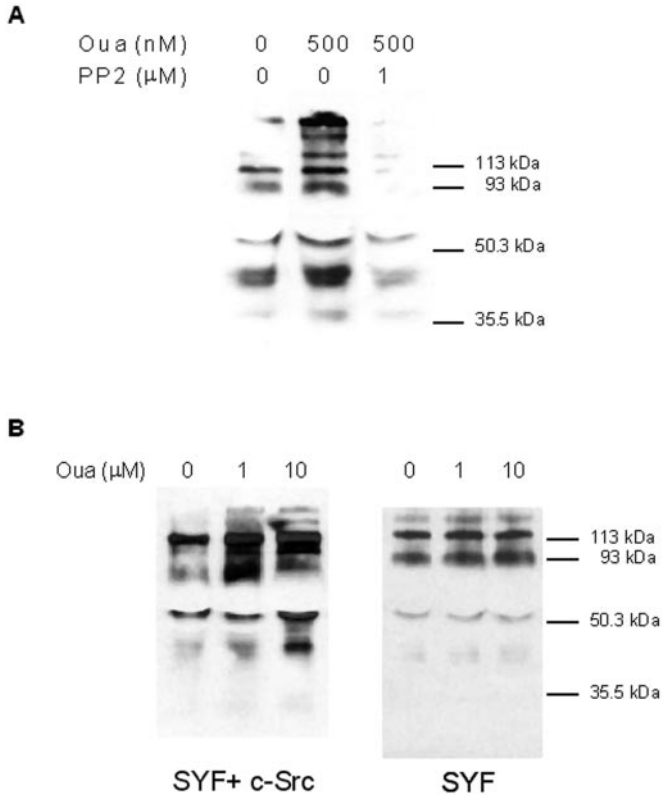

FIG. 5. **Src mediates the ouabain-induced tyrosine phosphorylation in the isolated caveolae.** *A*, aliquots of the caveolae prepared form LLC-PK1 cells were pretreated with 1 μM PP2 for 15 min. Both control and PP2-pretreated caveolae were then exposed to 500 nM ouabain for 5 min. Proteins were precipitated by trichloroacetic acid, and tyrosine phosphorylation was assayed as in Fig. 4*B*. *B*, caveolae purified from SYF and SYF+c-Src cells were treated with indicated concentrations of ouabain and assayed for tyrosine phosphorylation as in *A*. These experiments were repeated four times.

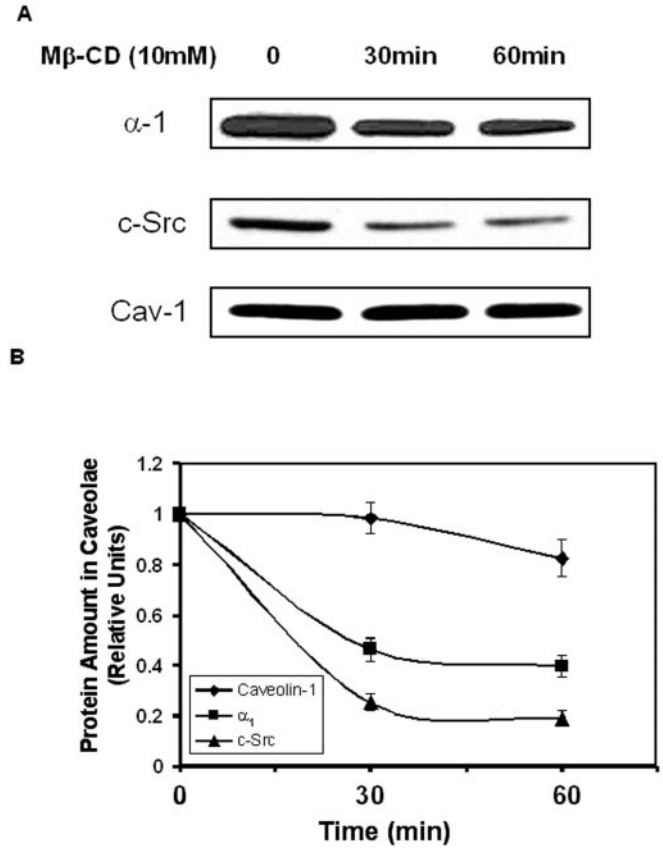

FIG. 6. **Effects of cholesterol depletion on the caveolar signaling proteins.** LLC-PK1 cells were treated with 10 mM Mβ-CD for either 30 or 60 min. Caveolae were isolated, and probed for caveolin-1, c-Src and the α_1 subunit as in Fig. 4*A*. *Panel A* shows a representative Western blot. *Panel B* shows the quantitative data from four independent experiments. Values are mean ± S.E. and are expressed relative to a control value of 1.

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 caveolae structure. Indeed, as illustrated in Fig. 4*B*, ouabain caused a dose-dependent increase in tyrosine phosphorylation of several proteins in the isolated caveolae as it did in live cells. Consistently, when caveolae 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. 4*D* and Ref. 10).

As shown in Fig. 4*A*, membrane fractions 8 and 9 also contain a significant amount of Na^+/K^+-ATPase and Src. To demonstrate that the Na^+/K^+-ATPase in caveolae, but not in other membrane fractions, responds to ouabain stimulation, we repeated the experiments depicted in Fig. 4*B* 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. 4*C*). These findings are consistent with the notion that the Na^+/K^+-ATPase may have to be preassembled in caveolae with its partners to function as a signal transducer.

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

Depletion of Either Cholesterol or Caveolin-1 Moves Na^+/K^+-ATPase and Src Out of Caveolae—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 in LLC-PK1 cells. To further test the functional role of caveolae in ouabain-activated signal transduction, LLC-PK1 cells were treated with 10 mM Mβ-CD for different times to deplete cholesterol from the plasma membrane. Because depletion of cholesterol has been shown to disrupt the structure of caveolae (23–28), we reasoned that Mβ-CD might affect the ability of the cell to concentrate the Na^+/K^+-ATPase and its signaling partners in caveolae. Indeed, Western blot analysis showed that, although Mβ-CD had marginal effects on caveolin-1 content in the isolated caveolae, it caused more than 50% reduction in the caveolar Na^+/K^+-ATPase and Src (Fig. 6). Because the total protein recovered in the caveolae fraction was not altered by cholesterol depletion (data not shown) as previously reported (23), and Mβ-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.

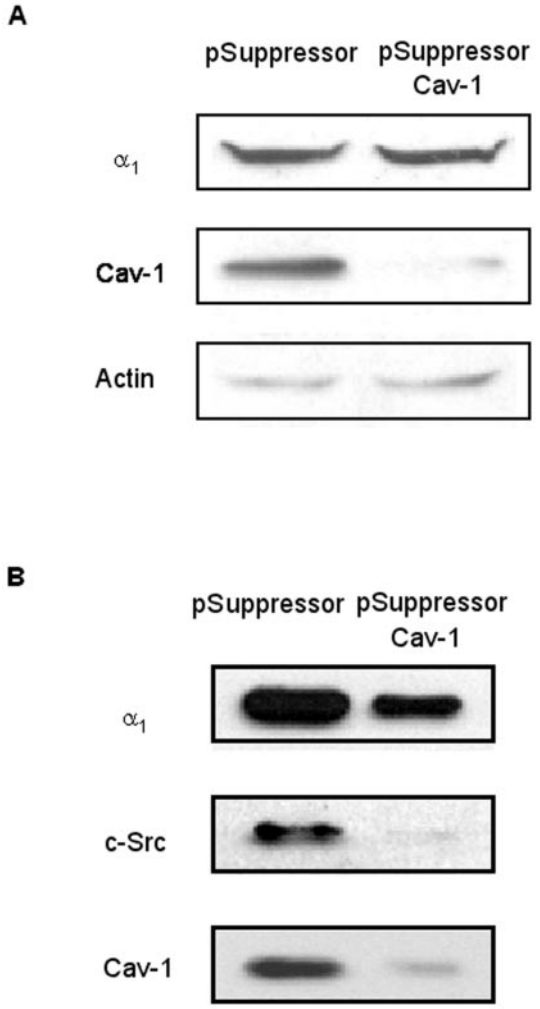

FIG. 7. **Depletion of caveolin-1 reduces caveolar Na^+/K^+-ATPase and Src.** 293 cells were transfected with either pSuppressor empty vector or pSuppressor cav-1. The cells were selected with puromycin, and puromycin-resistant colonies were cloned and expanded as described under "Experimental Procedures." *A*, a Western blot analysis of total cell lysates shows that expression of caveolin-1-specific siRNA depletes cellular caveolin-1 but not the Na^+/K^+-ATPase and β-actin. *B*, caveolae (fractions 4 and 5) were isolated from either pSuppressor or pSuppressor cav-1 cells and analyzed for the indicated proteins by Western blot. The experiments were repeated four times. α_1, α_1 subunit of Na^+/K^+-ATPase; *Cav-1*, caveolin-1.

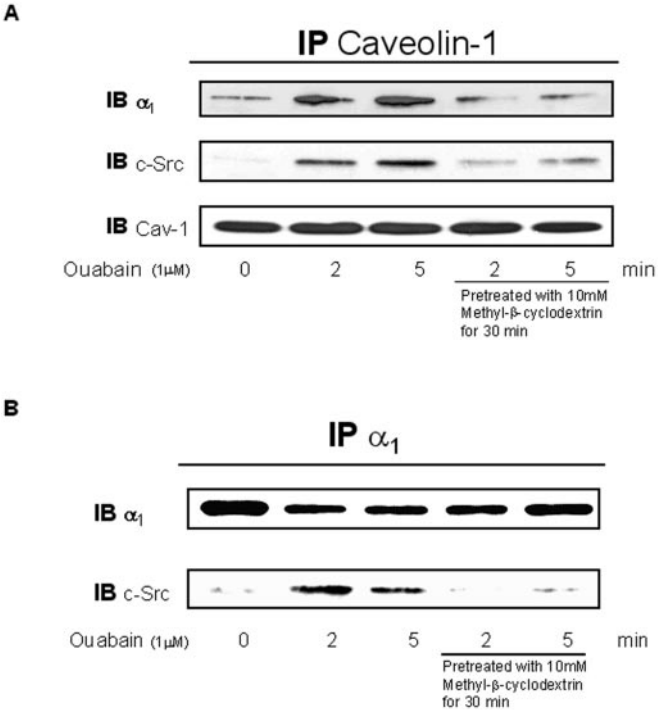

FIG. 8. **Depletion of cholesterol disrupts ouabain-induced interaction among Na^+/K^+-ATPase, c-Src, and caveolin-1.** Cells were treated with or without 10 mM Mβ-CD for 30 min. Cell lysates were immunoprecipitated with either anti-caveolin-1 (*A*) or anti-Na^+/K^+-ATPase α_1 subunit (*B*) antibodies, and the immunocomplexes were analyzed by Western blot for the α_1 subunit, c-Src, and/or caveolin-1. A representative blot of four independent experiments is shown for each panel. α_1, the α_1 subunit of Na^+/K^+-ATPase; *cav-1*, caveolin-1.

1 and 2), we reasoned that this interaction might be important for clustering of the signaling pump with its partners in caveolae. To test this hypothesis, we first constructed an expression vector for human caveolin-1-specific siRNA. Control experiments showed that one of the constructs (C2) reduced total cellular caveolin-1 in 293 cells when the cells were transiently transfected with the expression vector. Therefore, we made a stable cell line with the C2 vector. Cells transfected with empty vectors were also selected and used as a control. As shown in Fig. 7*A*, expression of C2 siRNA significantly reduced caveolin-1 content (12 ± 4% of control, $n = 4$), but had no effect on the Na^+/K^+-ATPase (93 ± 6%, $n = 4$) in 293 cells. Interestingly, depletion of caveolin-1 also reduced the amount of the Na^+/K^+-ATPase and Src in the isolated caveolae (Fig. 7*B*).

Based on the data presented in Figs. 4 and 5, we reckoned that reduction of the caveolar Na^+/K^+-ATPase should prevent ouabain-induced assembly of the signaling Na^+/K^+-ATPase-Src-caveolin-1 complex, because decreases in local concentrations of these proteins could prevent them from interacting with each other. Indeed, when cell lysates were immunoprecipitated by an anti-caveolin-1 antibody, depletion of cholesterol by Mβ-CD blocked ouabain-induced association of Src and Na^+/K^+-ATPase to caveolin-1 (Fig. 8*A*). Furthermore, when cell lysates were immunoprecipitated by an anti-α_1 antibody, we found that depletion of cholesterol also abolished ouabain-induced recruitment of Src to the signaling Na^+/K^+-ATPase (Fig. 8*B*). Because the formation of the Na^+/K^+-ATPase-Src complex is essential for ouabain-induced activation of Src and other signaling proteins, the above findings led us to examine the effects of Mβ-CD on ouabain-induced activation of ERK1/2. As depicted in Fig. 9*B*, Mβ-CD significantly inhibited ouabain-induced activation of ERKs. On the other hand, Mβ-CD did not inhibit the effects of EGF on ERKs under the same experimental conditions (Fig. 9*A*). To confirm the effects of Mβ-CD on ouabain-induced ERK activation are due to removal of cholesterol, cholesterol-depleted LLC-PK1 cells were replenished with cholesterol in the presence of Mβ-CD, and then exposed to ouabain. As shown in Fig. 9*C*, cholesterol repletion restored ouabain-induced ERK1/2 phosphorylation. To substantiate the above findings, we also determined the effects of ouabain on ERKs in caveolin-1-depleted 293 cells. As depicted in Fig. 9 (*D* and *E*), although ouabain activated ERKs in a time-dependent manner in control cells, the effect was partially blocked in the caveolin-1-depleted 293 cells. These results indicate that the interaction of Na^+/K^+-ATPase with caveolin-1 is important for clustering the pump with its signaling partners in caveolae, which is essential for the ouabain-activated signaling events.

DISCUSSION

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

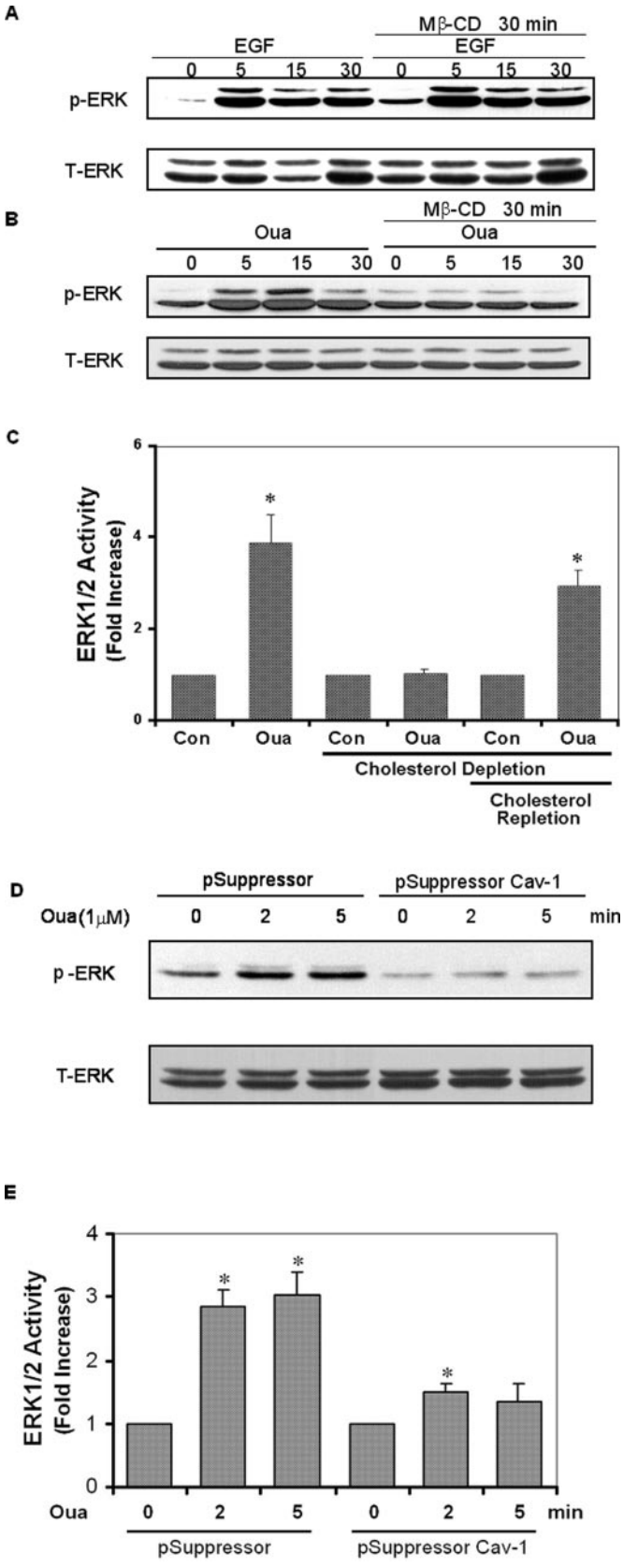

FIG. 9. *A–C*, effects of cholesterol depletion/repletion on EGF or ouabain-induced ERK activation. LLC-PK1 cells were incubated with 10 mM Mβ-CD for 30 min, and both control and Mβ-CD-treated cells were exposed to 50 ng/ml EGF (*A*) or 1 μM ouabain (*B*) for 5, 15, or 30 min. *C*, after cholesterol depletion with Mβ-CD, cells were repleted with cholesterol as described under "Experimental Procedures." Both cholesterol-depleted and -repleted cells were treated with 1 μM ouabain for 15 min. Cell lysates were analyzed by Western blot for phosphorylated 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.

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.* Φ*XX*Φ*XXXX*Φ and Φ*X*Φ*XXXX*Φ), 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. 1*A*), 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. 1*B*). 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-

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 cholesterol 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 progesterone 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. 8*A*). 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. 9*A*), 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-α_1 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 repletion 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 signal 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.

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