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# Role of caveolae in ouabain-induced proliferation of cultured vascular smooth muscle cells of the synthetic phenotype

**Lijun Liu,[1] Joel Abramowitz,[2] Amir Askari,[1] and Julius C. Allen[2]**
[1]*Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43614; and* [2]*Section of Cardiovascular Sciences, Department of Medicine, Baylor College of Medicine, Houston, Texas 77030*

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**Liu, Lijun, Joel Abramowitz, Amir Askari, and Julius C. Allen.** Role of caveolae in ouabain-induced proliferation of cultured vascular smooth muscle cells of the synthetic phenotype. *Am J Physiol Heart Circ Physiol* 287: H2173–H2182, 2004. First published July 15, 2004; doi:10.1152/ajpheart.00352.2004.—We have shown earlier that low concentrations of ouabain that do not perturb the ionic milieu can initiate proliferation of vascular smooth muscle cells (VSMCs) in the synthetic phenotype from three different species: canine, rodent, and human. This effect occurs by activation of Src and the epidermal growth factor receptor (EGFR), and thus supports the concept of an additional, nonionic, transducing function of the Na pump. The present study presents data suggesting that such activation occurs through specific Na pump sites localized to the caveolae, and subsequent interactions with selected signaling proteins resident within the same membrane microdomain. Our data show that at rest, 30% of the total number of Na pumps are concentrated within the caveolae. When the various VSMCs were treated with proliferating concentrations of ouabain, the key protein content in isolated caveolae was increased. However, the recruited proteins were different between the different tissues. Thus ouabain activated the recruitment of both the Na pump $\alpha_1$-subunit and EGFR in the caveolae from rat A7r5 cells, whereas in both human and canine cells, ouabain activated the recruitment of Src, with the caveolar content of the other proteins remaining constant. These data demonstrate that ouabain interacts with the $\alpha_1$-subunit of the Na pump that resides within the caveolar domain, and such interaction selectively recruits signal transducing proteins to this microdomain resulting in their activation, which is necessary for the initiation of the proliferative cascade.

Na pump; ouabain signaling; vascular smooth muscle proliferation

THE NA PUMP maintains normal ion gradients across the cell membrane of virtually all mammalian cells, and the mechanism of ion transport has been studied extensively for many years (7, 21). Recently, however, it has become apparent that pump function can be modulated by a variety of interacting proteins with mechanisms other than enzymatic phosphorylation of PKA and PKC. Such protein modulators are the FXYD family of proteins, i.e., phospholemman (15, 16), the $\gamma$-subunit (28), and corticosteroid hormone-induced factor (16), which have been known for some time but whose functions have only recently been delineated. Their interaction with the Na pump can alter affinity for both ATP and ions (42).

However, these are not the only protein moieties that can interact with the pump. Recently, a number of studies have appeared indicating that the pump can also interact with proteins generally associated with the well-known growth-related transducing cascades. The functional manifestation of these pump-protein interactions are quite tissue specific (see below), and a common bond between all of these observations is that they are initiated by the binding of the cardiac glycoside inhibitor ouabain to the pump. However, the resulting effects appear to be separate and distinct from its well-known inhibitory effect, which results in changes in cytoplasmic ion levels.

Thus when low concentrations of ouabain interact with the pump, a variety of tissue-specific responses have been documented that are totally unrelated to ion turnover. For example, in cardiac cells, such interaction results in activation of an hypertrophic pathway via Src activation and transactivation of the epidermal growth factor receptor (EGFR) (17, 27, 32), and in both vascular smooth muscle cells (VSMCs) (1, 24) and kidney epithelial cells (12), such activation results in proliferation. Activation of the latter cells can also result in Ca oscillations due to the interaction of the $\alpha_1$-subunit of the Na pump with the inositol (1,4,5)-trisphosphate receptor (2, 29).

We have shown that very low concentrations of ouabain (0.1–1.0 nM in both canine and human VSMCs and 1.0 $\mu$M in rat A7r5 cells, reflecting the affinity of the $\alpha_1$-subunit of the Na pump in each tissue) can activate proliferation via Src/EGFR and ERK1/2 activation (1, 3). At these ouabain concentrations, there is no measurable effect on cytoplasmic ion levels. Certainly, although 1–2% of the resident pumps would be bound and inhibited by ouabain, leading to local increases in Na levels, there would be sufficient numbers of normally (unbound) functioning pumps available, so that the ion effect would be small and transient. Thus we proposed that because so few pump proteins had to be activated by these low levels of ouabain to achieve this proliferative effect that there may be a compartmentalization of pump localization that would facilitate pump interaction with the cascade proteins necessary for initiating the response. Because it has been shown in a variety of cells that caveolar microdomains act as scaffolding regions that effectively localize proteins that normally would not be in close enough proximity to interact (6, 35), we investigated the possibility that pump localization within caveolae might represent an important manifestation of this compartmentalization.

We have recently shown that there is a specific colocalization of the $\alpha_1$-subunit of the pump and caveolin-3 in cardiac muscle suggesting that this interaction may mediate the ouabain-induced cardiomyocyte hypertrophic response (27). Such specific clustered pump localization could allow low concentrations of ouabain to interact in a normal manner with the pump, but because of its specific location within the

Address for reprint requests and other correspondence: J. C. Allen, Section of Cardiovascular Science, Dept. of Medicine, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 (E-mail: juliusa@bcm.tmc.edu).

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caveolar structure would allow subsequent pump interaction with neighboring proteins normally not available for such interactions, when situated in the bulk plasma membrane (48). Thus the hypothesis of the present set of experiments is a logical extension of the data obtained in our earlier study but delineates a different end point for the newly found ouabain-initiated transduction function of the Na pump. This study describes experiments demonstrating ouabain-induced translocation into the caveolar microdomain and subsequent activation of key proteins in cultured VSMCs in the synthetic phenotype (46) under specific circumstances in which we have earlier shown initiation of a proliferative cascade rather than the hypertrophic cascade that occurs by such ouabain activation in cardiac tissue.

## MATERIALS AND METHODS

*Materials.* Chemicals of the highest purity and culture media were purchased from Sigma (St. Louis, MO). Anti-caveolin-1 monoclonal antibody (clone C060), anti-caveolin-1 rabbit polyclonal antibody, anti-caveolin-2 monoclonal antibody (clone 65), anti-caveolin-3 monoclonal antibody (clone 26), and anti-clathrin heavy chain monoclonal antibody were obtained from BD Transduction Laboratories (Lexington, KY). Anti-$Na^+$-$K^+$-ATPase-$\alpha_1$ monoclonal antibody, anti-Src (clone GD11) monoclonal antibody, polyclonal anti-EGFR antibody, and rabbit anti-sheep IgG-horseradish peroxidase (HRP) secondary antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies against ERK1/2(K-23), phosphorylated ERK1/2(E-4), actin (c-11), goat anti-rabbit IgG-HRP secondary antibody, and goat anti-mouse IgG-HRP secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against $Na^+$-$K^+$-ATPase-$\alpha_1$ ($\alpha$6F) was obtained from Developmental Studies Hybridoma Bank, the University of Iowa (Iowa City, IA).

*Cell preparation and culture.* A7r5 cells (American Type Culture Collection) were cultured in DMEM supplemented with 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 the experiments. For the confocal microscopy study, cells were seeded on laminin-coated coverslips.

Canine saphenous vein (CSVSMCs) and human umbilical vein SMCs (HUVSMCs) were prepared and cultured as described previously (1, 5). Research involving animals adhered to the American Physiological Society's "Guiding Principles in the Care and Use of Animals." All research on animals was done by Institutional Animal Care and Use Committee-approved guidelines.

Because CSVSMCs and HUVSMCs were cultured and passed, they represented the synthetic and not the contractile phenotype (46).

*Fractionation of cell lysates for caveolae preparation by carbonate-based procedures.* Caveolae isolation was conducted as described in our previous study (27) by the widely used carbonate-based and detergent-free procedure of Song et al. (40). Briefly, after being serum starved for 24 h, cells were either treated with ouabain or not. Three 150-mm dishes of 80–90% confluent cells (A7r5 cells, CSVSMCs, and HUVSMCs) were scraped, collected, and suspended 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 at 4°C. 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 20-s bursts). Two milliliters of 90% sucrose prepared in 25 mM MES (pH 6.5) plus 150 mM NaCl (MBS) was then added to the homogenate. 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 in a rotor (model SW41, Beckman) at 39,000 revolutions/min for 16–18 h, and twelve 1-ml fractions (numbered from top to bottom) were collected. The opaque caveolar membrane band was at the interface of 5% and 35% sucrose (*fractions 4* and *5*).

*Immunofluorescence microscopy.* After treatment, immunofluorescent staining of cells was conducted as described in our previous study (27). Briefly, cells were fixed with −20°C methanol and probed overnight at 4°C with anti-$Na^+$-$K^+$-ATPase-$\alpha_1$ monoclonal antibody (Upstate Biotechnology, 1:50) and anti-caveolin-1 polyclonal antibody (1:200) in goat serum dilution buffer, followed by Alexa Fluor 488-conjugated anti-rabbit IgG (H+L) (1:100) and Alexa Fluor 546-conjugated anti-mouse IgG (H+L) (1:100) antibodies in GSDB. The coverslips were mounted with Vectashield mounting medium (Vector Laboratories; Burlingame, CA). Laser scanning confocal microscopy was performed with a Bio-Rad Radiance 2000 (Bio-Rad; Hercules, CA). Images were captured using a ×60 water objective and transmitted to a personal computer with the software program LaserSharp 2000 (Bio-Rad). All fluorescence images were acquired by using excitation lines from an Ar laser at 488 nm and a green HeNe laser at 543 nm and emission filters of HQ515/30 for green and HQ590/70 for red. Image acquisition was done in the *x*-, *y*-, and *z*-dimensions with *z* steps of 0.50 μm for all fluorescence images. Fluorescent signals of both fluorochromes were recorded simultaneously at one scan. No cross talk was observed. The magnification, laser iris, gain, and offset parameters were optimized for each laser and were kept constant for all images. Digitalized image information was visualized using Adobe Photoshop (San Jose, CA).

*Immunoblot analysis.* Samples were subjected to 10% or 4–12% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences), and probed with appropriate antibodies by standard procedures. The immunoreactive bands were detected using enhanced chemiluminescence (Pierce). For quantitative comparisons, images were scanned with a Bio-Rad 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.

*Measurement of phosphorylation/activation of ERK1/2.* Immunoblotting was performed to identify the activation of ERK1/2 using antibodies that react with activated ERK1/2 and total ERK1/2. Dilutions of these antibodies were done as recommended by the manufacturer. After the indicated treatment of cells, the incubation medium was rapidly replaced with ice-cold PBS. The washed cells were then lysed in 200 μl of ice-cold modified radioimmune precipitation buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 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 *g* for 15 min, and supernatants were used for Western blot analysis. The membranes were probed with anti-phosphorylated ERK1/2 monoclonal antibody. After being stripped, the membranes were reprobed with a polyclonal antibody that recognizes the total amount of ERK1/2. The membranes were probed with anti-actin antibody to insure equal loading.

## RESULTS

*Colocalization of $Na^+$-$K^+$-ATPase subunits and related signaling proteins in SMC caveolae.* We have shown earlier that caveolae isolated by detergent-free procedures from both cardiac cells and kidney cells (HEK-293 and LLCPK1 cells) contain $Na^+$-$K^+$-ATPase (27, 48). In addition, two reports successfully used the same detergent-free method to isolate caveolae from vascular smooth muscle (19, 44). Thus we applied the same detergent-free fractionation procedure to isolate the caveolae from CSVSMCs, HUVSMCs, and A7r5 cells because this clearly was successful with previous prepa-

rations. The resulting fractions were then assayed for protein content and subjected to Western blot analyses for the three caveolin isoforms and the other indicated proteins. The typical distribution pattern for total protein and the localization of key proteins is shown for A7r5 cells (Fig. 1). Detection of the three isoforms of caveolin in A7r5 cells is in agreement with previous findings reported on the caveolin isoform content in VSMCs (19). The caveolar fractions (*fractions 4* and *5*) contained ~1–2% of the total cellular protein.

Because it had been suggested that detergent-free isolation of caveolin-rich fractions may contain extraneous membrane constituents (39, 41), we also probed these isolated fraction for clathrin, shown by others (36, 49) to be exluded from caveolae. Clearly, there was complete separation of the three caveolin isoforms in *fractions 4* and *5* and clathrin, which was contained in the heavier *fractions 10–12*.

Typical distribution patterns for caveolin-1, the $Na^+$-$K^+$-ATPase $\alpha_1$-subunit, and related signaling proteins in fractionated samples of CSVSMCs and HUVSMCs are shown in Fig. 2. The total protein distribution pattern for these two VSMC sources was the same as with the A7r5 cells (data not shown). Nearly identical patterns were obtained after fractionation of all three cell types. The $\alpha_1$-isoform of the catalytic subunit of $Na^+$-$K^+$-ATPase is the only isoform found in these sources of VSMCs (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 these data, and the pattern of distribution of total protein in the 12 fractions (Fig. 1), we conclude that, whereas both the caveolin-rich light fractions (*fractions 4* and *5*) and the heavy fractions (*fractions 8–12*) contain substantial quantities of the $\alpha_1$-subunit, *fractions 4* and *5* are significantly enriched in the $\alpha_1$-subunit relative to the heavy *fractions 8–12* per unit protein.

Although the experiments shown Figs. 1 and 2 showed the presence of significant amounts of the $Na^+$-$K^+$-ATPase $\alpha_1$-isoform and several related signaling proteins in caveolae isolated from the VSMC lysates, it was necessary to make a more 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 shown in 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

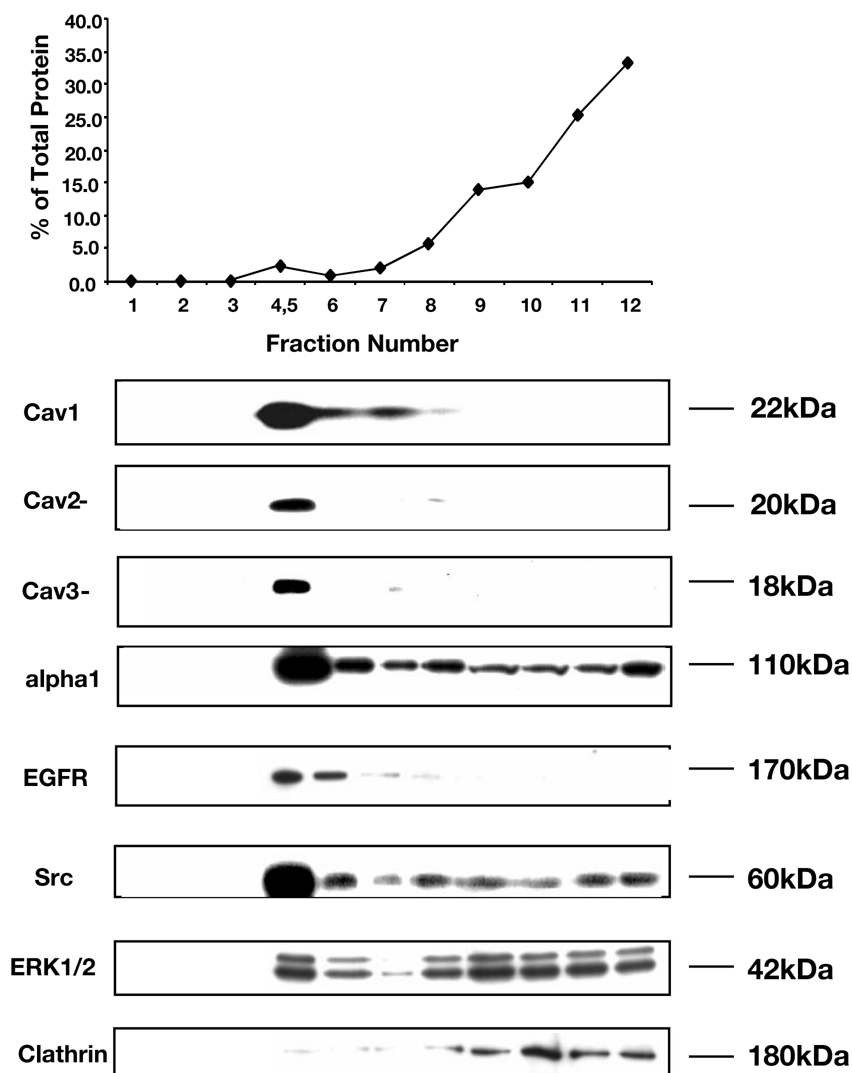


Fig. 1. Presence of the $\alpha_1$-isoform of $Na^+$-$K^+$-ATPase and related signaling proteins in caveolae prepared from the lysates of A7r5 cells. Cell lysate was prepared and fractionated by the carbonate-based procedure, and 12 fractions of equal volume (1 ml each) were collected as described in MATERIALS AND METHODS. *Fractions 4* and *5* were combined for analyses because they contained the opaque caveolar membrane band located at the 5–35% sucrose gradient interface. Distribution of the total lysate protein in the various fractions is shown on *top*. *Fractions 1–3* did not contain protein. An equal amount of protein (10 μg) from each fraction was subjected to SDS-PAGE and Western blot analysis with the indicated specific antibodies (*bottom*). Cav, caveolin; EGFR, epidermal growth factor receptor.

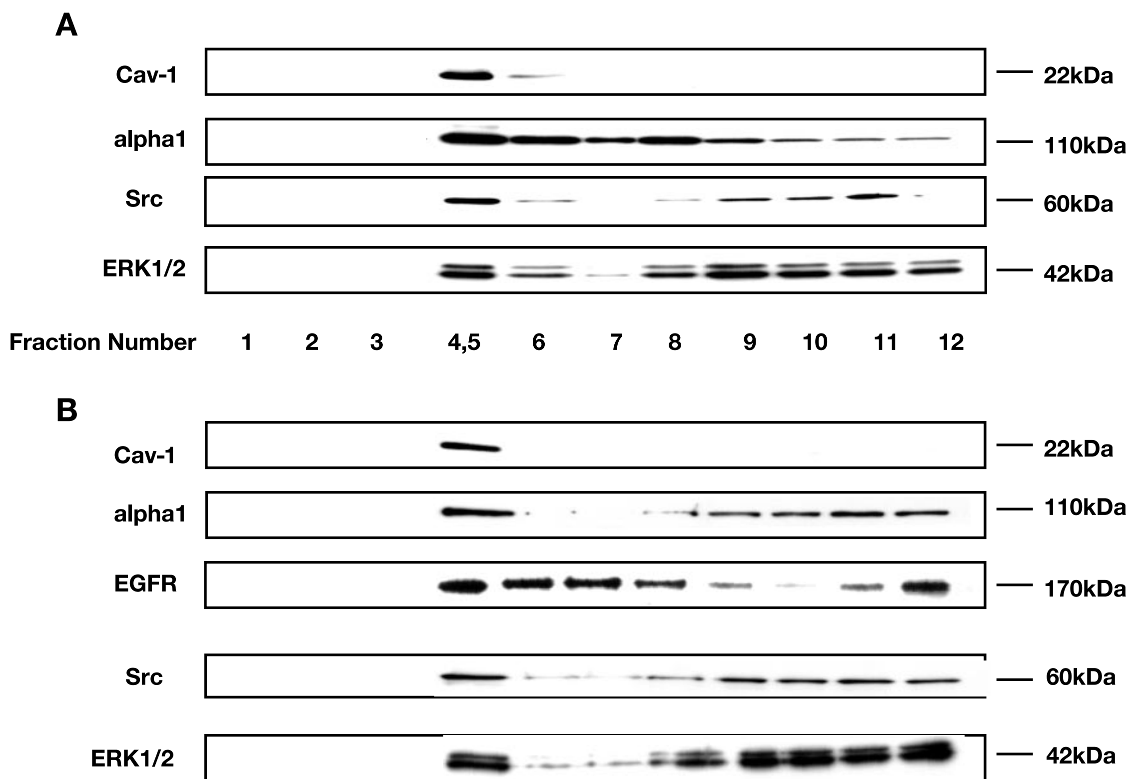


Fig. 2. Presence of the $\alpha_1$-isoform of $Na^+$-$K^+$-ATPase and related signaling proteins in caveolae prepared from canine (*A*) and human vascular smooth muscle cells (*B*). Lysates of the primary cultures of the indicated cells were fractionated and analyzed as described in Fig. 1. Protein distribution patterns and Cav-2 and -3 blots (not shown) were similar to those shown in Fig. 1.

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. 3 for A7r5 cells) by using samples from cell lysates. 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. The caveolar content of each protein, as a percentage of the total, was then calculated. The results of these experiments are summarized in Fig. 3 and show the caveolar contents of caveolin-1, -2, and -3, the $\alpha_1$-subunit, EGFR, Src, and ERK1/2. These data indicate that ~30% of total cellular content of the $\alpha_1$-isoform is located in caveolae. The remainder must be contained within the bulk plasma membrane and the internal membranes.

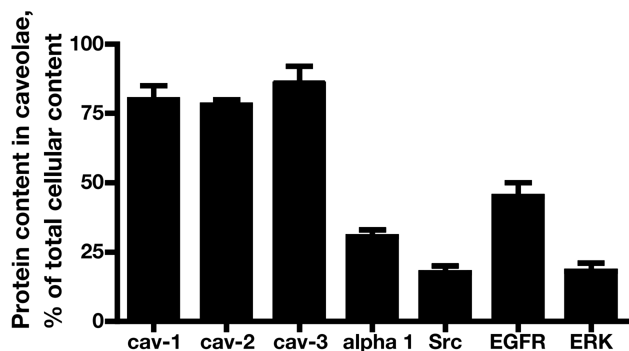


Fig. 3. Relative distributions of Cav-1, -2, and -3, $\alpha_1$-isoform of $Na^+$-$K^+$-ATPase, EGFR, Src, and ERK1/2 in caveolar and noncaveolar fractions of A7r5 cell lysates. Fractionations were performed as in Fig. 1 on samples from 2 different preparations. Immunoblots of the indicated proteins 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. The indicated values are means and ranges of the values obtained from the 2 independent experiments.

We used immunostaining and confocal microscopy to explore the subcellular localizations of caveolin-1 and the $\alpha_1$-isoform of $Na^+$-$K^+$-ATPase in A7r5 cells (Fig. 4). Immunostaining showed the presence of both the $\alpha_1$-isoform and caveolin-1 proteins throughout the cell, both on the cell membrane and within the cytoplasm, presumably in internal membranes. Such data are consistent with the observations in the literature for both proteins. It has been shown (43, 46) that the caveolar localization within SMCs is not limited to the plasma membrane and changes significantly as the cells remain in culture. This dynamic caveolar distribution within VSMCs has more recently been demonstrated by Vallejo and Hardin (46) and Kawabe et al. (22). The former workers showed that there was a shift in the predominant caveolin distibution from the membrane to the cytoplasm in A7r5 cells as the cells remained in culture. However, despite this relative shift, both areas still retained significant caveolin. Because the A7r5 cells used in this study were cultured as well, they must also represent the synthetic and not the contractile phenotype (46).

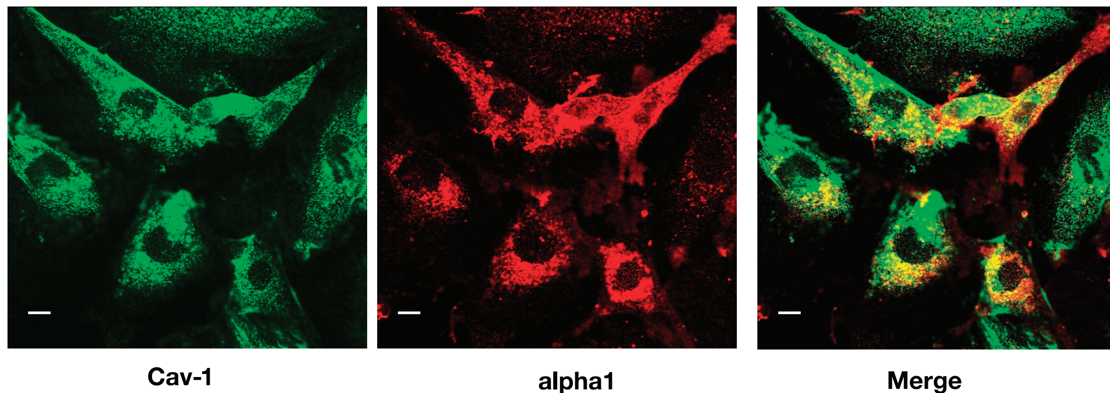


Fig. 4. Colocalization of the $Na^+$-$K^+$-ATPase $\alpha_1$-isoform and Cav-1 in A7r5 cells. Cells were fixed for immunostaining and confocal microscopy (see MATERIALS AND METHODS). Red, staining of $\alpha_1$; green, staining of Cav-1. Scale bar = 10 μm.

In addition, the Na pump has been shown to be dispersed within the cytoplasmic inclusions and internal membranes. Indeed, we (24) have shown that intact Na pumps translocate to the cell membrane of VSMCs when the cells require more pumps in the membrane, when grown in a low-K (pump inhibiting) medium. What is important in the present context is that our findings indicated considerable overlap in the immunostaining of the $\alpha_1$-subunits and caveolin-1. The combined data shown in Figs. 1–4 strongly support a suggested possible colocalization of caveolin and a significant portion of $Na^+$-$K^+$-ATPase in VSMCs. Importantly, even though the Na pump and caveolin-1 appear to interact within the cytoplasm, there are sufficient pumps localized within the caveolae at the membrane to interact with ouabain to activate the cascade.

*Ouabain effects on vascular caveolar pools of $Na^+$-$K^+$-ATPase and related signal transducing proteins.* We (23) have recently shown that the increase in proliferation of CSVSMCs by low concentrations of ouabain (1 nM) is accompanied by the activation of the proximal signaling events leading to the activation of Src and ERK1/2. In the relatively ouabain-resistant A7r5 cells, 1 μM ouabain induces proliferation comparable to that obtained with 1 nM ouabain in CSVSMCs (1, 3). It is most important to note that it has been clearly shown that this concentration of ouabain has no effect on the Na pump of A7r5 cells or rat vessels, so there would be no significant alteration of cytoplasmic ion content (8, 9). The results of the experiments shown in Fig. 5 show that in these A7r5 cells, 1 μM ouabain also induced significant activation of ERK1/2. We conclude, therefore, that in both ouabain-resistant and ouabain-sensitive VSMCs, ouabain-induced proliferation is preceded by the rapid activation of signaling events leading to ERK1/2 activation.

However, these data do not, by definition, suggest the involvement of an intact structure of the caveolar domains. The compound β-methyl-cyclodextrin has been shown to deplete membrane cholesterol (14, 47). Thus if treatment of VSMCs would prevent the specific ouabain activation of ERK1/2, this would certainly lend credence to the suggestion that ouabain is activating the cascade via an interaction with a specific population of $\alpha_1$-subunits. Thus we treated A7r5 cells with 10 μM β-methyl-cyclodextrin for 30 min before the addition of ouabain. The resulting data are shown in Fig. 6. It is clear that, although such treatment itself activates ERK1/2, it attenuated the activation of ERK1/2 by ouabain, suggesting the necessity of intact caveolae for the manifestation of the ouabain effect.

In the following experiments (shown in Figs. 7–10), we exposed A7r5 cells to 1 μM ouabain and CSVSMCs and HUVSMCs to 0.1–1.0 nM ouabain, prepared caveolae from treated and untreated cells, and determined the caveolar contents of caveolin-1, the $\alpha_1$-subunit, and the indicated signaling proteins.

In A7r5 cells, the results (Fig. 7*A*) showed that ouabain treatment did not change the caveolar contents of caveolin-1 or Src but that the caveolar contents of EGFR and $\alpha_1$-subunit of

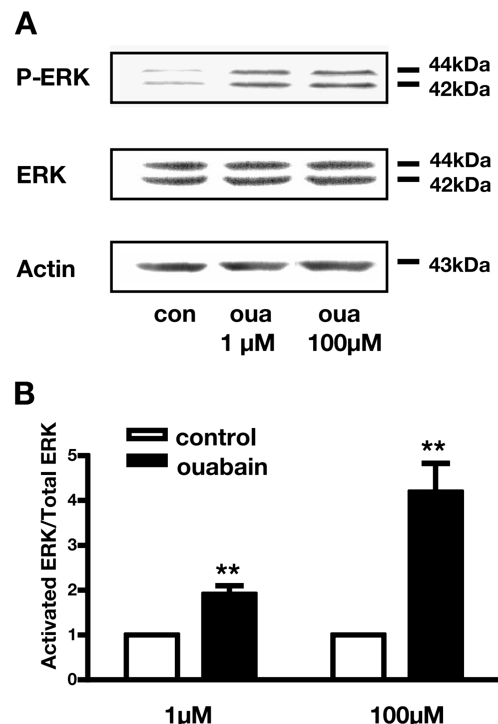


Fig. 5. Ouabain-induced activation of ERK1/2 in A7r5 cells. Cells were treated with 1 or 100 μM ouabain (Oua) for 5 min. Activation of ERK1/2 was assayed as described in MATERIALS AND METHODS. *A*: representative Western blot on ouabain-induced activation of ERK1/2. *B*: ratio of phospho-ERK1/2 (p-ERK) to total ERK1/2 relative to the same ratio in untreated control (Con). The indicated values are means ± SE of 4 experiments. $^{**}P < 0.01$ (*t*-test).

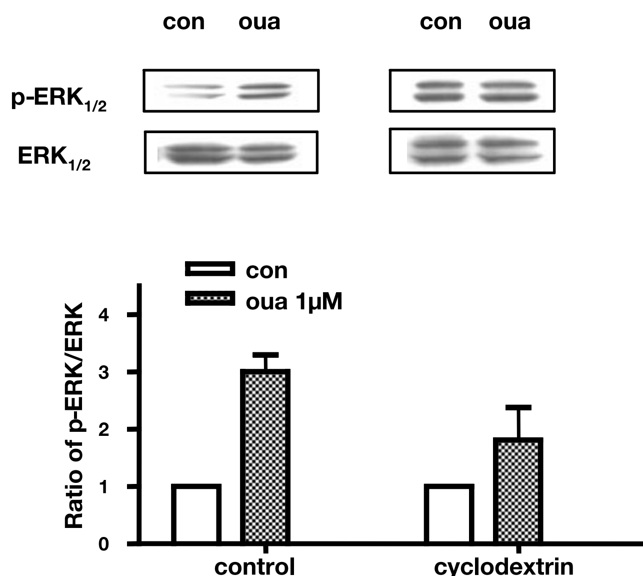


Fig. 6. Effects of treatment of cultured A7r5 cells with β-methyl-cyclodextrin on ouabain-induced activation of ERK1/2. After serum starvation, A7r5 cells were incubated with DMEM or 10 mM β-methyl-cyclodextrin for 30 min and were then treated with the indicated ouabain concentrations or control medium for 15 min. The cells were lysed with RIPA buffer containing inhibitors. Western blots of A7r5 cell extracts were probed with a monoclonal antibody (Santa Cruz Biotechnology, E-4) to phosphospecific ERK1/2 and a polyclonal antibody against ERK1/2 (Santa Cruz Biotechnology, K23). Active ERK1/2 was normalized for total ERK1/2 loading on the gel and expressed as the fold increase from ouabain-untreated levels. The indicated values are means ± SE of duplicate experiments.

$Na^+$-$K^+$-ATPase were significantly increased. Figure 7*B* expands the Western blots shown in Fig. 7*A*, with multiple experiments. There was a statistically significant increase in the caveolar localization of both the $\alpha_1$-subunit and EGFR at 5 and 15 min of ouabain treatment.

Confocal images also suggested that the merging of caveolin-1 and the $\alpha_1$-subunit of $Na^+$-$K^+$-ATPase in A7r5 cells was increased by ouabain treatment in the same manner (Fig. 8). The control cells showed some merging of the $\alpha_1$-subunit and caveolin signal, which by visual inspection appears to be increased after 5 min of ouabain treatment. Although a statistical analysis of these data has not been completed, repeated experiments have demonstrated the same results. These confocal images are supportive of our contention that ouabain alters the protein interaction, but additional, more quantitative assessment is necessary before any definitive conclusions can be reached.

In similar experiments to those shown for A7r5 cells shown in Fig. 7, the effects of 0.1 and 1.0 nM ouabain on both canine and human cells show results that are quite different from those obtained with the rat cells (Figs. 9 and 10). We used these low concentrations of ouabain and the short time periods of 5 and 15 min to coincide with the concentrations and times that we have already shown provide maximal stimulation of ERK1/2 in canine VSMCs, a sensitive species (3, 23). Thus Src was the only altered caveolar protein in these two cell types at both ouabain concentrations, whereas the other proteins remained constant. Ouabain treatment appears to activate recruitment of selected proteins to the caveolae in these different VSMCs. However, the same proteins are not recruited in each cell type (compare Fig. 7 with Figs. 9 and 10).

## DISCUSSION

Four previous studies have indicated that low ouabain concentrations can activate SMC proliferation: one on prostate SMCs (10) and ours on canine, human, and rat VSMCs (1, 5, 24). These recent studies have suggested that the mechanism of ouabain activation occurred through protein-protein interactions that had been previously shown for cardiac muscle and kidney epithelial cells (17, 27, 47). However, the cascade activates an hypertrophic pathway in cardiomyocytes and a proliferative pathway in the kidney cells. The data presented in the present study further suggest that the ouabain-induced proliferative effects observed in three different sources of VSMCs can be explained at least in part by a specific drug interaction with the Na pump component localized to the caveolar microdomain.

It is important to note that we have earlier shown the same ouabain proliferative effect in cultured VSMCs that contain either ouabain-sensitive (human and canine) or ouabain-resistant A7r5 cell (rat origin) Na pumps at concentrations that reflect the resident $\alpha_1$-subunit's relative sensitivity to ouabain (1). Thus the effective range of ouabain concentrations for the sensitive species was 0.1–10 nM and for the resistant species exactly three orders of magnitude higher. The 1,000-fold difference in the effective concentration is strong, albeit indirect, evidence that this proliferative effect is initiated through ouabain binding to the $\alpha_1$-subunit of the pump and not some

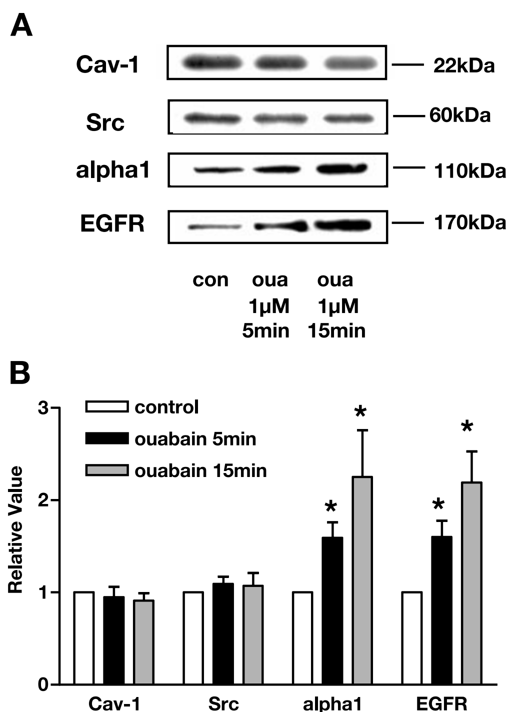


Fig. 7. Effects of ouabain treatment on the caveolar contents of Src, EGFR, and $\alpha_1$-isoform of $Na^+$-$K^+$-ATPase in A7r5 cells. Cells were exposed to indicated concentrations of ouabain for 5 or 15 min (see MATERIALS AND METHODS). Samples of control and treated cells were fractionated as in Fig. 1, and caveolae (*fractions 4* and *5*) were quantitated for indicated proteins. $*P < 0.05$ compared with control (*t*-test). Representative Western blots are shown in *A*, and quantitation is shown in *B*. The number of pairs of control and ouabain-treated caveolar preparations immunoassayed for the indicated proteins were as follows: Cav-1, $n = 5$; $\alpha_1$-subunit, $n = 6$; EGFR, $n = 6$; and Src, $n = 5$.

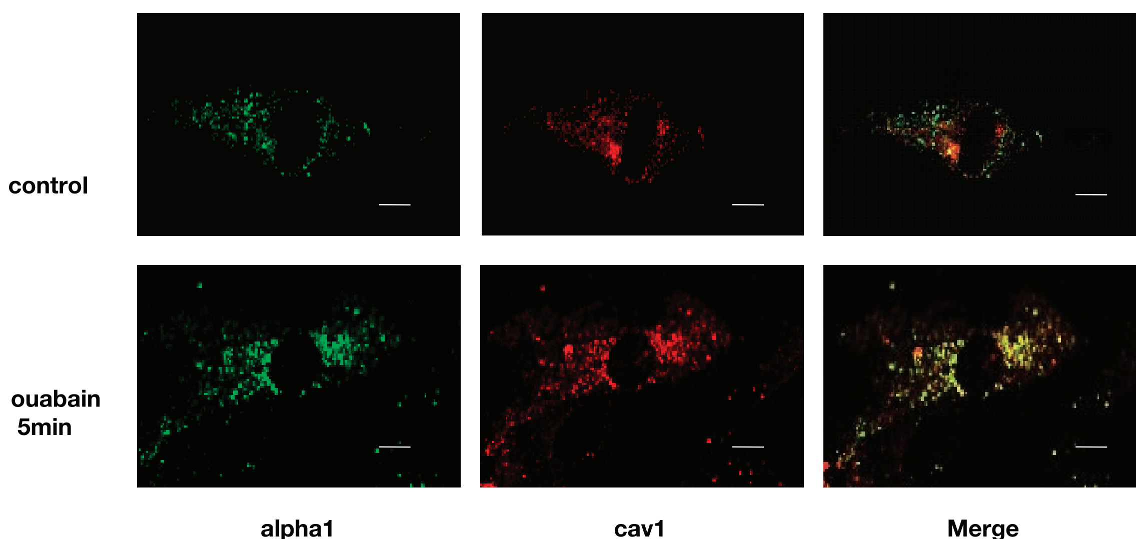


Fig. 8. Confocal images of representative A7r5 cells after ouabain treatment. The methods were the same as in Fig. 4 except the cells were treated with 1 μM ouabain as indicated. Green, staining of $\alpha_1$-subunit; red, staining of Cav-1. Experiments were performed independently 3 times with similar results. Scale bar = 10 μm.

extraneous, as-yet-unidentified protein. Although ouabain binding to the Na pump from a variety of tissues is a well-known observation (4, 5), this present effect occurs at concentrations below which traditional ligand binding studies are easily performed.

Figure 2 clearly shows that all the transducing proteins that we have shown to participate in the ouabain-activated VSMC proliferation pathway reside within the caveolae in all three VSMC sources. Whereas this is certainly not unexpected for proteins such as Src and EGFR, the finding of $Na^+$-$K^+$-ATPase in caveolae requires further discussion because of the conflicting reports on the caveolar localization of this enzyme. The presence of the Na pump in caveolae was first suggested based on indirect evidence (30). However, a number of previous studies concluded that caveolae/rafts that are prepared biochemically from several cell types do not contain $Na^+$-$K^+$-ATPase (13, 18, 26, 37, 49). Caveolar fractions may be prepared either by detergent-free procedures or those that use Triton X-100 or other detergents (6, 40). It is now well established that the use of detergents in such procedures may cause the loss of some normal caveolar residents (6, 40). In our recent work on cardiac caveolae, we (27) established the

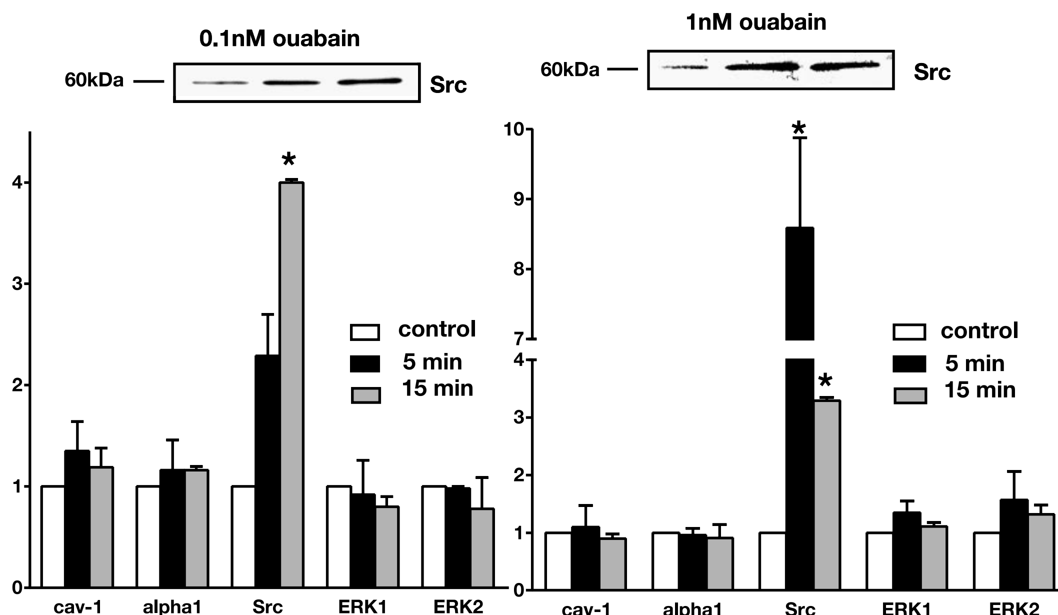


Fig. 9. Effects of ouabain treatment on the caveolar contents of signaling proteins in canine saphrenous vein smooth muscle cells. Experiments were performed as indicated in Fig. 7 and MATERIALS AND METHODS. Representative blots are shown at *top* and quantitation of such blots from experiments on multiple samples is shown at *bottom*. The indicated values are means ± SE of 3 experiments. Data were subjected to one-way ANOVA, and statistical significance was assessed using the Bonferroni *t*-test. $*P < 0.05$ compared with control.

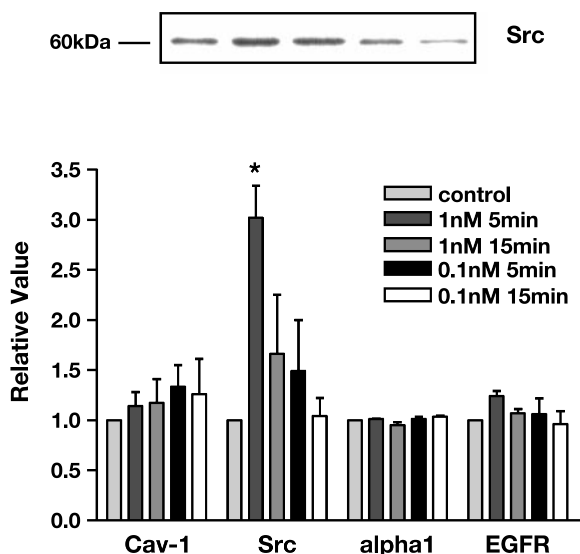


Fig. 10. Effects of ouabain treatment on the caveolar contents of signaling proteins in human umbilical vein smooth muscle cells. Experiments were performed as indicated in Fig. 7 and MATERIALS AND METHODS. Representative blots are shown at *top* and quantitation of such blots from experiments on multiple samples is shown at *bottom*. The indicated values are means ± SE of 3 experiments. Data were subjected to one-way ANOVA, and statistical significance was assessed using the Bonferroni *t*-test. $*P < 0.05$ compared with control.

caveolar localization of $Na^+$-$K^+$-ATPase with the use of two different detergent-free fractionation procedures and by the immunopurification of the caveolae. We (27) also showed that $Na^+$-$K^+$-ATPase is indeed among those caveolar residents removed by Triton X-100, and in all likelihood the previous failure of others to find the Na pump in caveolae was due either to the use of this detergent or the inadequate sensitivity of the assays used for its detection. We now have demonstrated in this study that deteregent-free isolation of caveolae from A7r5 cells excludes the noncaveolar protein clathrin, making it highly unlikely that the $\alpha_1$-subunit is also a noncaveolar protein and that its appearance within this microdomain is due to contamination occurring during the isolation procedure.

It is clear, however, that there still is considerable controversy in the literature regarding which procedure best isolates caveolae. In a recent review, Stan (42) has clearly stated an appropriate approach: "From this confusion stems again the conclusion that there is no perfect cell fractionation method and the results obtained with each of the existing ones need validation by at least one additional independent approach" (see also Ref. 39). It is for this reason that we used two separate isolation procedures and immunopurification in the previous study (27) and confocal microscopy in both that study and the present one to support the contention of caveolar localization of the Na pump.

The present results (Fig. 2) now provide further support for the caveolar localization of the Na pump by showing the presence of a significant fraction of the total cellular content of the enzyme in caveolae from three different sources of cultured VSMCs. Indeed, the quantitative estimation of the caveolar content in one of these cells (Fig. 3) shows that about 25% of total cellular $Na^+$-$K^+$-ATPase is in caveolae. Interestingly, this is about the same proportion as seen in cardiac cells (27), despite the fact that the density of Na pump sites in cardiac muscle membrane is much higher than in any VSMC membrane (4). An important validation of this localization of the Na pump to caveolae is the fact that we have confirmed (Fig. 1) that a well-known protein, clathrin, normally not present in this microdomain in other tissues, is also excluded from the caveolar fraction isolated from A7r5 cells.

Although the detailed mechanism of the interactive effect of ouabain on these cells still awaits clarification, a most important aspect of the process can be seen in Figs. 7–10 showing the differential effect of incubation with proliferative concentrations of ouabain in canine and human VSMCs (0.1–1.0 nM) and rat VSMCs (1.0 μM). In each case, there is a translocation of selected proteins into the isolated caveolar region, although different proteins move in different cell species. Most importantly, all are proteins that we have shown to be associated with ouabain-induced proliferation (23).

These differential response data raise an important question regarding the need for recruitment of key proteins in response to specific ligands that act through caveolar localization. It appears that not all proteins are present at sufficient levels to allow them to interact, and selected proteins must be recruited into the caveolar domain to interact with the proper moiety to complete the pathway. Thus it appears that the content of selected proteins may limit the process, and not all proteins that are necessary for the complete pathway may reside in the structure until activated. The concept of recruitment of key proteins to the caveolae has received much attention (31, 36). Lamb et al. (25) have suggested that the human bradykinin $B_2$ receptor appears to "shuttle" its agonist through the caveolar domain, whereas the $B_1$ receptor seems to reside there by default. Ishizaka et al. (19) have shown that angiotensin II affects movement of the angiotensin II type 1 receptor into caveolae in rat VSMCs. Such a function(s) for the $\alpha_1$-subunit of the Na pump (the ouabain receptor) is currently under investigation for this pathway as well.

Currently, we do not know the significance of the differential effect of ouabain on the recruitment process in the three tissue sources. The preparations are from three different species, and from three different muscle sources, but the specific recruited proteins appear to reflect the sensitivity of the $\alpha_1$-subunit of the Na pump to ouabain. Thus in both the human and canine cells (high sensitivity), only Src is recruited, whereas in ouabain-resistant A7r5 cells, the $\alpha_1$-subunit and EGFR are both recruited. It is tempting to speculate that because of the high sensitivity of the pump in canine and human cells that it is unnecessary to recruite additional pumps to the caveolae to complete the signaling module, whereas in the resistant species, it is necessary to recruit more proteins to activate the signaling process. Because of the very low concentrations at which ouabain can activate VSMC proliferation in human and canine VSMCs, it is intriguing to consider the possibility that this effect might in some way be related to the functions of endogenous digitalis-like factor (EDLF). Although these compounds have been discussed for many years, and are finally recognized by most workers in the field as being "real," their functional role is still uncertain (11, 34). One of the major complications through many years has been that the levels at which these factors have been measured in vivo have been well below that which can show clear signs of pump inhibition. However, the concentrations at which we have

observed these proliferative effects in both human and canine VSMCs are similar to those measured in vivo by a variety of workers (34, 38). In addition, we have also shown that marinobufagenin, another putative EDLF, has the same effect on these VSMCs as does ouabain (1, 3).

It is clear that ouabain can activate a proliferative pathway in at least three different VSMC sources, and it has recently been shown that it can activate the same phenomenon in rat kidney epithelial cells as well, also at very low concentrations (12). Our data are consistent with the hypothesis that the interactive protein moieties that mediate this effect are contained within the caveolae but certainly do not prove this paradigm. Additional work such as defining the specific protein-protein interactions that are altered in response to ouabain must be characterized before definitive conclusions can be reached.

Recent data from a variety of laboratories have implicated caveolae in the control of both mitogenic signaling (44) and contractile regulation (20) in vascular smooth muscle. These specific microdomains are taking on more significance in these tissues as has already been suggested for cardiac muscle and endothelial cells.

Thus the present data support the growing concept that the Na pump complex has functions in addition to its better-known ion transport regulatory role. These functions are mediated by specific ouabain-induced interactions of the Na pump with specific transducing proteins. Indeed, although the mechanism of the well-known fundamental ion transport homeostatic function seems to be similar in all tissue, the transduction function activated by low noninhibiting levels of ouabain seems to be far more tissue specific in its ultimate effect, which may well reflect different in vivo tissue functions. The variability in this molecular response (e.g., adult cardiomyocytes do not proliferate, but VSMCs do) may relate to the specific molecular and cellular properties of the different cell types.

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Present address of J. Abramowitz: Div. of Intramural Research, National Institutes of Environmental Health Sciences, PO Box 12233, Mail Drop: A2-09, Research Triangle Park, NC 27709.

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