The large conductance, voltage- and Ca\(^{2+}\)-activated potassium (MaxiK, BK) channel and caveolin-1 play important roles in regulating vascular contractility. Here, we hypothesized that the MaxiK α-subunit (Slo1) and caveolin-1 may interact with each other. Slo1 and caveolin-1 physiological association in native vascular tissue is strongly supported by (i) detergent-free purification of caveolin-1-rich domains demonstrating a pool of aortic Slo1 co-migrating with caveolin-1 to light density sucrose fractions, (ii) reverse co-immunoprecipitation, and (iii) double immunolabeling of freshly isolated myocytes revealing caveolin-1 and Slo1 proximity at the plasma membrane. In HEK293T cells, Slo1-caveolin-1 association was unaffected by the smooth muscle MaxiK β1-subunit. Sequence analysis revealed two potential caveolin-binding motifs along the Slo1 C terminus, one equivalent, "1007YNMLCFGIY1015", and another mirror image, "537YTEYLSSAF545", to the consensus sequence, "εXXXXεXXε". Deletion of "1007YNMLCFGIY1015" caused ~80% loss of Slo1-caveolin-1 association while preserving channel normal folding and overall Slo1 and caveolin-1 intracellular distribution patterns. "537YTEYLSSAF545" deletion had an insignificant dissociative effect. Interestingly, caveolin-1 coexpression reduced Slo1 surface and functional expression near 70% without affecting channel voltage sensitivity, and deletion of "1007YNMLCFGIY1015" motif obliterated channel surface expression. The results suggest "1007YNMLCFGIY1015" possible participation in Slo1 plasmalemmal targeting and demonstrate its role as a main mechanism for caveolin-1 association with Slo1 potentially serving a dual role: (i) maintaining channels in intracellular compartments downsizing their surface expression and/or (ii) serving as anchor of plasma membrane resident channels to caveolin-1-rich membranes. Because the caveolin-1 scaffolding domain is juxtamembrane, it is tempting to suggest that Slo1-caveolin-1 interaction facilitates the tethering of the Slo1 C-terminal end to the membrane.

Large conductance, voltage- and Ca\(^{2+}\)-activated potassium (MaxiK, BK)\(^4\) channels play important roles in vascular, neuronal, and urinary functions. In vascular smooth muscle, MaxiK channel appears to be a unique signaling protein because of its ability to mediate the effects of several vasoconstricting as well as vasodilating agents. The ability of MaxiK protein to complete with high fidelity these opposite tasks calls for specific associations and subcellular compartmentalization with corresponding signaling partners (1). Recently, it has been appreciated that many signaling molecules are segregated primarily in specialized microdomains like caveolae (plasma membrane invaginations enriched with cholesterol and caveolin protein), thereby, optimizing signal transduction between agonists and specific effectors (2).

Three caveolin proteins have been identified, caveolin-1, -2, and -3. All of them seem to be expressed in smooth muscle (3, 4). However, gene ablation experiments have shown that caveolin-1 plays a major role in the vasculature and pulmonary function. In this animal model, the disappearance of caveolae (and caveolin-1) uncouples MaxiK channel activity to Ca\(^{2+}\) sparks (3), and there is an increased channel current density at the surface membrane (5). Yet, mechanisms favoring the interaction of MaxiK pore-forming α subunit (Slo1) with caveolin-1 or explaining an increased expression in the absence of caveolin-1 are missing. Mammalian Slo1 possesses two potential motifs for caveolin-1 binding: one consensus ("1007YNMLCFGIY1015") and another mirror image ("537YTEYLSSAF545") to the consensus motif, "εXXXXεXXε" (where ε is an aromatic amino acid and X is any amino acid) (6). The presence of these two sites makes Slo1 an excellent target for interaction with caveolin-1. We found for the first time that native Slo1 from rat aorta tightly associates with caveolin-1 sharing similar microdomains and displaying a close proximity at the surface of isolated vascular myocytes. We also found that the caveolin-1-binding motif "1007YNMLCFGIY1015" in Slo1 is the main mechanism underlying Slo1-caveolin-1 interaction and represents a new venue for channel surface expression control.

\(^{4}\)The abbreviations used are: MaxiK, large conductance, voltage- and Ca\(^{2+}\)-activated K\(^{+}\) channel; Slo1, α-subunit of MaxiK channel; Cav-1, caveolin-1; SD, scaffolding domain; WB, Western blot; IP, immunoprecipitation; MES, 2-(N-morpholino)ethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Ab, antibody; PPI, protein proximity index; CC, correlation coefficient; FPo, fractional open probability.

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EXPERIMENTAL PROCEDURES

Materials—Affinity-purified Abs were polyclonal anti-Slo1 (anti-Kv1.1, Alomone Labs, lot AN05), polyclonal (N-20, Santa Cruz Biotechnology), and monoclonal (BD Transduction Laboratories) anti-caveolin-1, monoclonal anti-c-Myc (clone 9E10, Sigma), and polyclonal anti-β1 subunit (anti-Maxi Potassium channel beta, Novus Biologicals). Secondary Abs (goat) for cells were Alexa Fluor 560 or 594 antimouse and Alexa 488 anti-rabbit; for Western blot (WB) were Alexa Fluor 680 anti-rabbit (Invitrogen) and IRDye 800 antimouse (Rockland Immunochemicals). Human Slo1 (hSlo1) (U11058, KCNMA1) (with N-terminal c-Myc epitope) (7), and rat (r) caveolin-1 (AF439778) constructs subcloned in pcDNA3 (Invitrogen) or pIRES (Clontech) vectors were used; human β1 subunit (U25138, KCNMB1) was in pcDNA3. rCaveolin-1 and hSlo1 share near 100%, and hSlo1 shares 90% amino acid sequence homology with corresponding mammalian orthologues. Mutagenesis was performed with QuickChange (Stratagene) and confirmed by sequencing. Purified caveolin-1 scaffolding domain (SD) peptide was custom-made (Tufts University Core Facility).

Animals—Sprague-Dawley male rats (~200 g) were used. Protocols received institutional approval.

Antibody Properties—Anti-Slo1 recognized a single strong band of ~125 kDa in WB of transfected cells (Figs. 3 and 4; signals of dimers may also be detected); in native aorta, WB shows a strong signal of the expected molecular mass (~125 kDa; weak signals that may correspond to dimers may also be detected) and a weaker ~75-kDa band. WB and immunocytochemistry signals were all absent by preadsorbing the Ab with the antigenic fusion protein (1 μg of Ab:10 μg of antigen/ml). Anti-caveolin-1 Abs (poly and monoclonal) recognized a single strong band of the expected size (~21 kDa) in transfected cells (Figs. 3 and 4) and in aortic lysates and do not cross-react with expressed caveolin-2 or caveolin-3 (not shown). No signal was detected when polyclonal caveolin-1 Ab was preadsorbed with the antigenic peptide (0.25 μg of Ab:2.5 μg of peptide/ml, Santa Cruz Biotechnology). Anti-β1 subunit polyclonal Ab recognized a single strong band of ~26 kDa (corresponding to the partially glycosylated form; without glycosylation the β1 molecular size is ~22 kDa) in transfected cells (8) (Fig. 3), and the signal practically vanished when the Ab was preadsorbed with the antigenic peptide (2 μg of Ab:10 μg of peptide/ml, Novus Biologicals). Secondary Abs gave practically no signals in immunocytochemistry and WB.

Solubility Test—Aortas were extracted (~100 mg/200 μl) in (mm): 150 NaCl, 5 Tris-HCl, 100 NaF, 5 EDTA, 1 Na2VO4, 0.5 lodoacetamide, 10 HEPEs, pH 7.4, 0.1% Nonidet P-40, 0.25% sodium deoxycholate, plus protease inhibitors. Homogenates were centrifuged (3,300 g, 10 min, 4 °C), and the supernatant was precleared with 50 μg/ml protein-G-Sepharose beads (1 h, 4 °C) and centrifuged (2,500 g, 30 min, 4 °C), then with 25 μg of protein-G-Sepharose beads (1 h, 4 °C) and centrifuged (15,000 × g, 30 min, 4 °C). Precleared lysates (~1.5 mg of protein) were incubated with anti-Slo1 or anti-caveolin-1 polyclonal Abs (2 μg, 3 h), then with 25 μl of protein-G-Sepharose beads (overnight), washed (10% glycerol/lysis buffer), eluted with loading buffer (plus 1.4 μM β-mercaptoethanol), boiled, and subjected to WB. A similar procedure was used for HEK293T cells, but Abs were pre-bound to protein-G-Sepharose beads (2 μg of Ab/50 μl of beads, 1 h, 4 °C). WB signals (infrared fluorescence, LI-COR Biosciences) were measured as integrated pixel intensities with ImagePro or Metamorph.

Digital Image Processing—Confocal sections were acquired over 0.1–0.2 μm (z-axis) at 0.115 μm/pixel (x-y plane) for aortic myocytes and every 0.25 μm at 0.0575 μm/pixel for transfected cells. Images were three-dimensionally blind deconvolved using AutoQuant, and pixel intensities were measured with Metamorph. Analysis for protein proximity
**Slo1 Channel and Caveolin-1 Complex**

index (PPI) was carried out with custom-made software (11). All conditions, including optical sectioning and exposures, were identical for a given experiment.

**Patch Clamp**—Macrocscopic currents were measured in inside-out patches. To compare current densities, we used pipettes of similar resistances (~2 MΩ) that should have similar diameters and normalized the currents to the resistance of each patch pipette. Voltage-dependent activation (fractional open probability (FPO) – voltage) curves were obtained by fitting the experimental data to a Boltzmann distribution: \( \text{FPO} = \frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp[(V_{1/2} - V)\zeta \delta F/(RT)]} \), where \( G \) = conductance, \( G_{\text{max}} \) = limiting maximum conductance, \( V_{1/2} \) = half-activation potential, \( \zeta = \) effective valence, and \( F, R, \text{and } T \) have their usual thermodynamic meanings. Pipette and bath solutions were (mM): 105 potassium methanesulfonate, 5 KCl, 10 HEPES (free Ca\(^{2+} \) = 66 μM), pH 7.0. Caveolin-1 SD peptide was reconstituted in 70% ethanol (stock 10 mM). Its effect was tested by first acquiring control currents in the presence of vehicle alone (0.07% ethanol and 0.01% bovine serum albumin).

**Statistical Analysis**—Data are mean ± S.E. Tests for significance were with Student's t test (\( p \)) except in Fig. 2, where the normal approximation of the sign test (\( p_{\text{sign test}} \)) was used. \( p < 0.05 \) was considered significant; \( n \) denotes the number of preparations. A minimum of three different animals was used in all experiments.

**RESULTS**

**Association of Slo1 and Caveolin-1 in Native Vascular Tissue**—We first examined the possible association of Slo1 with caveolin-1 in native rat aorta by three independent methods: (i) co-migration in sucrose density fractions after detergent-free extraction; (ii) reverse co-immunoprecipitation (co-IP), and (iii) co-localization in freshly dissociated myocytes.

**Co-migration of Slo1 and Caveolin-1 in Sucrose Density Fractions**—We investigated whether Slo1 can share with caveolin-1 similar membranous structures that could be identified by their sedimentation properties. In particular, caveolin-1-enriched membrane domains (i.e. caveolae) are typically detergent-resistant and segregate into lighter sucrose density fractions (12). We found that, opposite to myometrium and cultured aortic endothelial cells (Refs. 13, 14 and our own observations with myometrium) but similar to colonic epithelial cells (15), aortic tissue extraction with 1% Triton X-100 resulted in complete solubilization of Slo1 and caveolin-1 with their partitioning to heavy sucrose density fractions (not shown). These variations in Triton X-100 solubilization profile could be the result of the lipid composition of each tissue (16) making caveolin-1 domains detergent-resistant in myometrium but not in aorta. To find proper solubilization-extraction conditions that could preserve aortic caveolin-1-enriched membranes (17–19), we performed solubility tests. With the exception of Na\(_2\)CO\(_3\) (insoluble/soluble ratio = 10 ± 3.5, \( n = 3 \)), 1% Triton X-100 and other detergents (see “Experimental Procedures”) readily solubilized caveolin-1 (mean insoluble/soluble ratio = 1.8 ± 0.2, \( n = 2–3 \) for each detergent) preventing the isolation of caveolin-enriched aortic membranes of light buoyancy. Thus, we used Na\(_2\)CO\(_3\) to avoid disruption of caveolin-1-enriched membranes of this blood vessel. Western blots (Fig. 1A) demonstrate that a population of Slo1 (upper panel, –125 kDa) co-migrates with caveolin-1 (middle panel, Cav-1, –21 kDa) to light sucrose-density fractions (12–27% sucrose) (\( n = 9 \)), whereas the non-caveolar marker, clathrin heavy chain (20), is predominant in the heavy fractions (\( n = 7 \)).

**Reverse Co-IP Supports Slo1 and Caveolin-1 Close Association in Native Vascular Tissue**—The co-migration profile in Fig. 1A suggests the possible association of aortic Slo1 and caveolin-1 in a macromolecular complex. Indeed, reverse Co-IP experiments using aortic lysates support this idea. To maximize the specificity of associations, Co-IP was performed under high stringency in the presence of detergents (0.1% Non-ident P-40 and 0.25% sodium deoxycholate). To differentiate caveolin-1 signals (~21 kDa) from huge signals of the IgG light chain (~25 kDa), IP and WB analyses were performed with anti-caveolin-1 Abs generated in two different hosts. Typical WB analysis (Fig. 1B) shows the signals of Slo1 and caveolin-1 in tissue lysates (lane 1), and the products after IP without (blank, lane 2) and with Abs against Slo1 (lane 3) and caveolin-1 (lane 4). Regardless of the Ab used for IP, Slo1 and caveolin-1 were pulled down together. The specificity of the co-IP results was attested by the lack of signals in the blank. The mean values for co-immunoprecipitated caveolin-1 using anti-Slo1 Ab indicate that about 13 ± 6% (\( n = 3 \)) of caveolin-1 was tightly associated with Slo1 in aorta (Fig. 1C). Likewise, 15% of Slo1 could be co-immunoprecipitated using anti-caveolin-1 Ab (\( n = 2 \)).

**Co-localization of Slo1 and Caveolin-1 in Freshly Dissociated Aortic Myocytes**—Because Slo1 is known to be abundant in smooth muscle, we directly analyzed the cellular distribution of Slo1 and caveolin-1 proteins in pre-cross-linked freshly dissociated aortic myocytes. These procedures were used to avoid any possible cellular remodeling due to enzymatic treatment or...
A lar in the absence or presence of planes of amyocyte labeled with anti-Slo1 polyclonal (Fig. 2, FEBRUARY 22, 2008 •)

vations as half of cells had a PPI preparations). Apparently there may be at least two cell populations as half of cells had a PPI > 0.5 (n = 8, with 5 cells PPI = 0.9), whereas the other half had PPI ≤ 0.5 (n = 8). To validate our measurements, Fig. 2 (H–J) illustrates the labeling (H) and quantitative analysis (I and J) of a cell labeled for the same protein (caveolin-1) but with two different Abs. Four planes at the middle of the cell were analyzed. As expected, PPI was ~1, and the majority of data points had a positive CC value with $p_{\text{sign test}} < 0.05$ (J, gray area). The mean PPI value was 0.98 ± 0.01 (n = 27 cells, two preparations).

Together the co-migration profile, the co-IP, and the significant degree of caveolin-1 and Slo1 co-localization in native aorta strongly support the idea that Slo1 can share similar plasma membrane domains with caveolin-1 and support the hypothesis that these two proteins may interact with each other. Thus, we designed experiments to test a potential protein-protein interaction and biological consequences.

Role of Smooth Muscle $\beta$1 Subunit in Slo1-Caveolin-1 Association in HEK293T Cells—Because in native vascular myocytes Slo1 is associated with its modulatory $\beta$1 subunit (22), we tested if Slo1 and caveolin-1 association is affected by coexpression of $\beta$1 subunit. Fig. 3A exemplifies the expression by WB of hSlo1, caveolin-1, and $\beta$1 subunit in samples used for IP with anti-caveolin-1 Ab; Fig. 3B illustrates that anti-caveolin-1 Ab is able to effectively co-IP hSlo1 independent of the absence (lane 1) or presence (lane 2) of $\beta$1 subunit. Although there was a
small tendency of hSlo1 reduction to only 13% when cells were co-transfected with hSlo1Δ1 and hSlo1-WT (Fig. 4D). Similar results were obtained using anti-c-Myc Ab (recognizing hSlo1 N terminus) for IP (not shown). Negative controls (IP with no Ab) did not show any signals supporting the specificity of the interactions.

To explore the role of the conserved aromatic amino acids (underlined) within 1007YNMLCFG1015 in hSlo1-caveolin-1 association, we mutated these residues to alanines. Interestingly, the point mutant hSlo1-Y1007A increased caveolin-1-hSlo1 association by ~40% (n = 7), whereas either mutant hSlo1-F1012A or hSlo1-Y1015A significantly decreased caveolin-1-hSlo1 interactions by ~15% each (n = 5). Their effects seemed to be additive as mutating the three aromatic residues to generate a triple-alanine mutant (hSlo1-Y1007A/F1012A/Y1015A) resulted in practically no change in caveolin-1-hSlo1 association (n = 5). These results indicate that residues Phe1012 and Tyr1015 (and possibly other residues within or around the motif) play a role in stabilizing Slo1 channel interaction with caveolin-1.

In contrast to the major role of 1007YNMLCFG1015 in hSlo1-caveolin-1 association, 537YTEYLSSAF545 contributes little, if any, to hSlo1-caveolin-1 association as its deletion (hSlo1Δ2) had practically no effect (Fig. 4E, n = 4). This result also speaks in favor of the specificity of 1007YNMLCFG1015 in mediating hSlo1-caveolin-1 association.

The reduced association between hSlo1Δ1 and caveolin-1 was further investigated to discern whether gross differential subcellular expression or hSlo1Δ1 misfolding could explain the results (Fig. 5). Because caveolin-1 is only accessible intracellularly, immunolabeling was performed in permeabilized cells co-expressing hSlo1-WT + caveolin-1 (A–C) and hSlo1Δ1 plus caveolin-1 (D–F). In both instances, caveolin-1 (green) and hSlo1 (WT or hSlo1Δ1) (red) distributed with similar patterns (Merge; C and F) with PPP1hSlo1-WT+caveolin-1 = 0.62 ± 0.04 (n = 42) and PPP1hSlo1Δ1+caveolin-1 = 0.53 ± 0.04 (n = 19). Thus, an overall dramatic change in subcellular distribution cannot explain the loss of hSlo1Δ1 and caveolin-1 interaction. In addition, hSlo1-WT and hSlo1Δ1 displayed identical sedimentation properties along the sucrose gradient (n = 4) and mostly shared the apoferritin (443 kDa) migration profile indicating that the majority are in the form of tetramers (~500 kDa) (Fig. 5, G and H). A similar profile was obtained for the deletion construct hSlo1Δ2 (not shown).

In summary, the results underscore the predominant role of 1007YNMLCFG1015 motif in mediating hSlo1-caveolin-1 interaction, because deletion of this motif in hSlo1 efficiently prevented their association by ~80–85%, which can not be explained by misfolding of hSlo1.
Caveolin-1 Controls hSlo1 Channel Surface and Functional Expression: Role of 1007YNMLCFGY1015—Caveolin-1 not only serves as a scaffold to keep together signaling molecules in caveolae but may also have functional consequences on their interacting protein partners by modulating their traffic in caveolar vesicles and/or inhibiting enzymatic protein activity (23, 24). Thus, we wondered whether caveolin-1 interaction with hSlo1 could affect hSlo1 surface expression and/or its voltage-dependent activation.

To allow simultaneous expression of hSlo1 + caveolin-1 in a given cell, a bicistronic vector containing both genes was used (Figs. 6 and 7). hSlo1 surface expression was monitored by immunolabeling live cells with anti-c-Myc Ab (against extracellular epitope, red) followed by permeabilization and double labeling of total hSlo1 protein with anti-Slo1 Ab (against C-terminal end epitope, green) (diagram in Fig. 6G) (10). The images reveal that hSlo1-WT surface expression was largely reduced in cells expressing hSlo1-WT + caveolin-1 (Fig. 6, A versus C). Remarkably, hSlo1Δ1-expressing cells showed no sign of surface labeling (Fig. 6E) but, after cell permeabilization, showed comparable expression (Fig. 6F) as hSlo1-WT in the absence (Fig. 6B) or presence (Fig. 6D) of caveolin-1 (levels ranged from 6.3 to 7.6 × 10^8 pixel intensity/cell). Co-expression of caveolin-1 does not rescue hSlo1Δ1 surface expression (not shown). Quantification of surface/total signals in parallel experiments indicates that hSlo1-WT surface expression was reduced by −60% in cells co-expressing hSlo1-WT + caveolin-1 and to background levels in cells expressing hSlo1Δ1. Surface/total expression of hSlo1-WT (n = 93) was set to 100%, and normalized values were for hSlo1-WT + caveolin-1 = 39 ± 6% (n = 34 cells) and for hSlo1Δ1 = 0.1 ± 0.01% (n = 78 cells) (Fig. 6G). In ∼10% of 135 cells examined that were transfected with hSlo1-WT + caveolin-1, hSlo1 was able to escape and surface expression was almost similar as in cells expressing hSlo1-WT alone.

As expected from the lack of obvious role of β1 subunit in caveolin-1-hSlo1 association, the β1 subunit did not affect caveolin-1-induced reduction of hSlo1 surface expression as well (Fig. 6H). Surface/total values in parallel experiments were 45 ± 6% (n = 33 cells) for hSlo1 + caveolin-1 and 45 ± 8% (n = 26 cells) for hSlo1 + caveolin-1 + β1 expressing cells when compared with hSlo1, which was set to 100% (n = 93 cells). Simultaneous live labeling of hSlo1 and β1 confirmed the expression of the β1 subunit.

The decreased surface expression of hSlo1 induced by caveolin-1 would predict decreased hSlo1 macroscopic currents (I) as I = iNPo, where i = unitary current, N = number of channels, Po = open probability. In agreement, inside-out patch clamp...
recordings show that macroscopic currents generated by hSlo1-WT alone were larger (Fig. 7A) than currents elicited in cells expressing hSlo1-WT + caveolin-1 (Fig. 7B). Consistent with immunocytochemistry data, hSlo1Δ1 did not generate any detectable currents upon stimulation with identical pulse protocols (Fig. 7C). Fig. 7D shows the mean current density as a function of voltage obtained for hSlo1-WT (open circles; n = 15, 3 cell transfections), hSlo1-WT + caveolin-1 (closed circles; n = 16, 3 cell transfections), or hSlo1Δ1 (closed triangles; n = 7, 2 cell transfections).

In contrast, hSlo1 voltage-dependent activation curves were unaffected by caveolin-1 co-expression with half activation potentials, \( V_{1/2}^{\text{hSlo1}} = -33 \pm 3 \text{ mV} \), \( n = 15 \) versus \( V_{1/2}^{\text{hSlo1} + \text{caveolin-1}} = -26 \pm 1 \text{ mV} \), \( n = 16 \) (intracellular \( \text{Ca}^{2+} \)/cytoplasmic \( \text{Ca}^{2+} \) were 6.6 \( \mu \text{M} \); \( p = 0.23 \)). Currents were also unaffected by perfusion of inside-out patches with 10 \( \mu \text{M} \) caveolin-1 scaffolding domain (caveolin-1 SD, \( ^{58}\text{DGIW/KASFTTFVTKYWFYR}^{101} \)), known to be responsible for the interactions between caveolin-1 and its partners (12). In this case, 10 \( \mu \text{M} \) caveolin-1 SD caused a non-significant shift of \( -1.2 \pm 1 \text{ mV} \) (\( n = 5 \), two cell transfections) in the voltage-activation curve. Current density quantification at conditions where the channels’ Po reach their limiting value (+80 mV and free \( \text{Ca}^{2+} \)) of 6.6 \( \mu \text{M} \) indicates that caveolin-1 can reduce the number of channels reaching the surface by an average of \( \sim 70\% \) (Fig. 7D, inset). Values were for: hSlo1-WT = 21 \( \pm \) 2.5 nA*M\( \Omega \) (\( n = 15 \)) and hSlo1-WT + caveolin-1 = 6.5 \( \pm \) 1.2 nA*M\( \Omega \) (13 out of 16 patches). In three cells, hSlo1-WT escaped the effect of caveolin-1 showing comparable expression to hSlo1-WT expressed alone; one cell exhibited a 98% reduction in current density mimicking hSlo1Δ1.

The complete loss of surface functional expression induced by the deletion of the consensus caveolin-1 binding domain in hSlo1Δ1 is independent of the cell type used for expression, because similar results were attained in oocytes injected with hSlo1Δ1 cRNA (\( n = 8 \), 2 oocyte batches).

The results, in particular the absence of hSlo1 surface expression when the hSlo1\(^{1007\text{YNNMLCFEGY}^{1015}}\) consensus caveolin-1-binding motif was deleted, together with biochemical experiments support the view that caveolin-1 association with hSlo1 may serve as a down-regulator of the channel surface expression.

After mild cross-linking, it is likely that these differences in protein proximity reflect true differences in types or metabolic states of vascular myocytes, rather than being a reflection of different degrees of cell transformation due to the cell isolation procedure, which can induce protein internalization (not shown). In this context, analyses of intimal and medial arterial myocytes in culture have revealed differences in contractile function and morphology supporting two different subpopulations of cells belonging to anatomically distinct regions of the rat aorta (25). Two differentially distributed Slo1 to caveolin-1 populations were also apparent from sucrose density fractionation because Slo1 was detected in both light (carbonate-resistant) and heavy (carbonate-solubilized) membrane fractions, whereas caveolin-1 was mostly present in light fractions. A bimodal distribution of Slo1 to caveolin-1 according to their solubilization properties also exists in human myometrial tissues (13). Supporting a subpopulation of aortic Slo1 in caveolin-1-rich microdomains are the co-IP results showing a fraction (\( \sim 20\% \)) of Slo1 tightly associated with caveolin-1, which coincides with the percentage (\( \sim 30\% \)) of double-immunolabeled cells showing a high degree of proximity between Slo1 and caveolin-1 proteins. The co-IP results also confirm the validity of the newly developed PPI method (11) to directly quantify the proximity of two proteins in native cells allowing the visual sorting of different populations of cells in regard to their macromolecular complexes.

**Mechanism of hSlo1 and Caveolin-1 Association**—Positive evidence for a functional correlation or association between Slo1 and caveolin-1 has been observed in other tissues (5, 13–15, 26). Pull-down experiments using glutathione S-transferase-caveolin-1 protein, and lysates of HEK293T cells expressing hSlo1 indicate that these two proteins can associate; however, an indirect binding cannot be ruled out (14). Direct binding of caveolin-1 to its partners is thought to occur via the juxtamembrane caveolin-1 SD (caveolin\(_{52-101}\)) with specialized motifs, \( \varphi \text{X} \varphi \text{XXX} \varphi \text{XX} \varphi \) (where \( \varphi \) and \( X \) are aromatic and any amino acid, respectively) localized in their binding proteins at sites that are cytoplasmically exposed (6). The direct interaction of these two complementary motifs has been confirmed in signaling proteins like G\(_{12\alpha}\) protein and receptor tyrosine kinases (e.g. epidermal growth factor and insulin.

**DISCUSSION**

**Association of Slo1 Protein with Caveolin-1 in Native Aortic Smooth Muscle Cells**—Figs. 1 and 2 conclusively demonstrate that, in native aortic tissue, caveolin-1 and Slo1 can be in close proximity to each other. Quantitative analysis of protein proximity in freshly isolated myocytes revealed that there may be at least two populations of cells characterized by their degree of proximity between Slo1 and caveolin-1 with \( \sim 30\% \) of cells showing a high degree of PPI (\( \geq 0.9 \)). Because dissociation of cells was performed...
receptors) (24, 27). Here, we report for the first time that hSlo1 and caveolin-1 interaction requires the consensus caveolin-1-binding motif YNMLCFGIY (28) in hSlo1 as most (~80%) of hSlo1-caveolin-1 association was lost following its deletion (Fig. 4). This could not be explained by a global misfolding or a major change in distribution pattern of the proteins (Fig. 5) even when hSlo surface expression was abolished (Fig. 6) as a significant amount of both hSlo1Δ1 and caveolin-1 co-localized intracellularly similarly to what was observed when hSlo1-WT and caveolin-1 were coexpressed (Fig. 5). A similar degree of interaction was reported for epidermal growth factor and insulin receptors ϕXϕXXXϕ motifs with caveolin-1 SD using in vitro peptide competition assays (27). Mutations of the conserved residues (ϕXXXϕXXϕ) to alanines further supported the role of YNMLCFGIY motif in hSlo1-caveolin-1 association, and highlighted the importance of neighboring Phe1012 and Tyr1015 in stabilizing caveolin-1 association by 15% each. Close by aromatic residues (ϕXϕ) have been shown to play a role in the association of glucagon-like peptide 1 receptor with caveolin-1 (28). To our surprise mutant Y1007A increased hSlo1-caveolin-1 association with hSlo1 suggesting that Tyr1007 may have a destabilizing effect per se on hSlo1-caveolin-1 association. Because the consensus sequences were obtained in vitro using short peptide sequences (6), it is reasonable to imagine that in native proteins conformational requirements are also important in caveolin-1-target interactions, and that additional residues within or outside the consensus motif may also play a role. A role for residues other than the conserved aromatic residues has been observed for the glucagon-like peptide 1 receptor (28). In any event, the mutational/deletion analysis reported here support the view that YNMLCFGIY motif plays a preponderant role in hSlo1-caveolin-1 interaction.

In addition to YNMLCFGIY, we also analyzed the role of a non-classic caveolin-1-binding motif, ϕXXϕXXXϕ (357YTEYLASSF) (354). We predicted that the site could be borne the remaining 20% of hSlo-caveolin-1 interaction. Close to our prediction, deleting this site resulted in a modest (~15%) but non-significant reduction in hSlo-caveolin-1 interaction. Interestingly, a sequence of this kind has been found to account for α-hemolysin-caveolin-1 interaction (29), which again points to the view that the structural context where the consensus sequence resides is also relevant for the efficiency of caveolin-1-target interactions. In any case, because caveolin-1 SD has a juxtamembrane localization, one can envision that its binding to its complementary motif in hSlo1 will “pin” the carboxy-terminal end of hSlo1 to the plasma membrane in such a way that this hSlo1 region would tether to the membrane internal leaflet. In summary, these findings indicate that Slo1 can associate with caveolin-1 using mainly as mechanism the Slo1 caveolin-1-binding motif YNMLCFGIY. Association of Slo1 with caveolin-1 SD (6) may provide an important structural means for Slo1 channel/signaling function.

New Role of Caveolin-1 in Controlling Surface Expression of hSlo1—Two main roles have been attributed to caveolin-1 in signal transduction: (i) to serve as a scaffold to keep together signaling molecules in caveolae and (ii) to serve as modulator of the activity of its associating partners (23, 24). Consistent with caveolin-1 serving as scaffold to keep Slo1 together with other signaling molecules our results showed that, in native rat aorta, caveolin-1 and a population of Slo1 are co-localized at the surface membrane and tightly associated, thus segregating to membranes with similar sedimentation properties (Figs. 1 and 2). However, our results in inside-out patches do not support a long term or direct role of caveolin-1 in modifying hSlo1 electrical properties as co-expression of caveolin-1 or perfusion of caveolin-1 SD peptide did not alter hSlo1 voltage-activation properties nor had an apparent effect on its kinetics. Our data are consistent with studies showing that the Ca2+/V sensitivities of Slo1 are similar in cerebral myocytes of caveolin-1−/− and caveolin-1 +/+ mice (5). Although caveolin-1 has an inhibitory effect on several associating partners (24), exceptions to this rule have been reported. Caveolin-1 SD increases the insulin receptor kinase activity (30), whereas caveolin-1 and caveolin-1 SD peptide have no effect on COX-2 enzyme activity, although caveolin-1 binds to it (31). Our present work extends these latter observations and supports the idea that caveolin-1 has no direct effect on hSlo1 channel voltage-activation properties.

Caveolins have also been reported to participate in the trafficking of two G-protein-coupled receptors to the plasma membrane, the angiotensin II type 1A receptor and the glucagon-like peptide 1 receptor (28, 32). In marked contrast to an important role of caveolin-1 in targeting G-protein-coupled receptors to the plasma membrane (32), our data indicate that efficient hSlo1 surface expression does not require co-expression of caveolin-1, but rather co-expression of caveolin-1 results in a reduction of the number of hSlo1 channels reaching the cell surface. Using two independent measurements, patch clamp and immunocytochemistry (Figs. 6 and 7), we discovered that co-expression of caveolin-1 with hSlo1 results in a reduction by ~60–70% in the number of channels reaching the surface. Our findings are consistent with recent reports showing that knocking down caveolin-1 protein by small interference RNA or gene ablation results in increased functional Slo1 (BK and KCa) current/channel density at the surface membrane (5, 14). Thus, we propose that caveolin-1 has another previously undescribed role, which is its ability to constitutively down-regulate Slo1 protein surface expression.

Role of hSlo1 Caveolin-1-interacting Motif YNMLCFGIY in hSlo1 Surface Targeting—We discovered that, besides serving for caveolin-1 interaction (Fig. 4), the YNMLCFGIY motif in hSlo1 carboxyl-terminal end is also required for surface targeting; its deletion keeps the hSlo1 channel trapped in intracellular compartments without affecting its tetrameric assembly (Fig. 5, G and H). The hSlo1 intracellular carboxyl-terminal end is known to play a role in Slo1 surface expression (33–35). The YNMLCFGIY sequence has several important features that may explain why its absence causes channel trapping inside the cell: (i) it has a di-hydrophobic motif (underlined), which may act as endoplasmic reticulum export signal (36), (ii) it has a targeting tyrosine-based motif YXXXψ (bold; ψ, any amino acid and ψ, hydrophobic residue), which is a general sorting sequence that directs proteins to various cellular compartments, including the plasma membrane (37), and (iii) it is located in a strategic position near another two sequences containing di-hydrophobic motifs,
Slo1 Channel and Caveolin-1 Complex

996YGKDFCALK1005 (in hSlo1, amino acids KD are replaced with DL, which add another dihydrophobic motif and a tyrosine-based motif) and 1047DYLIFCL1052, whose deletion is sufficient to prevent current development (38) and protein surface expression (35), respectively. 1047DYLIFCL1052 has been proposed to act as an export signal. It is reasonable to envision that the spatial conformation conferred by all three motifs provides a combinatorial signal with high affinity for coat proteins involved in the anterograde traffic of the channel protein. This assumption would explain why deletion of any of these strings of amino acids in the context of the whole protein causes a defect in surface expression.

The fact that Slo1 truncations containing the first 20 and 118 amino acids of the carboxyl-terminal end, Slo11–343 and Slo11–441 are reported to reach the membrane (39), regardless of the absence of motifs 996YGKDFCALK1005, 1047DYLIFCL1052 (35, 38), and 1007YNMLCFGIY1015 reported here, may be reconciled by the uncovering of alternate export signals in the truncation constructs, and support the idea that Slo1 traffic mechanisms require specific conformations of the C terminus.

Interestingly, deletion of 635YTEYLSSAF545 motif (hSlo1Δ2), although correctly folded, also prevents Slo1 surface targeting. However, the presence of this motif appears insufficient for Slo1 surface targeting (although it contains a dihydrophobic export signal); a truncation construct containing it but lacking C-terminal residues beyond amino acid 651, Slo11–651, has been reported not to reach the surface membrane, although it is correctly folded (34). Consistent with a major role of residues beyond 651, in the normal Slo1 traffic to the membrane, cotransfection of Slo11–651 with its complementary C-terminal region, Slo652–1113 (containing residues 996–1015 and 1047–1052) rescued the protein to the plasma membrane. Thus, it seems reasonable to propose that residues 996–1015 and 1047–1052 are relevant for normal channel surface expression.

Residues 996–1015 and 1047–1052 are encoded by the constitutive exons 26 and 27 of hSlo1 that seem to be selected through evolution for sequences whose overall structure are required for proper membrane targeting of the channel protein.

How can we explain the findings that hSlo1-WT + caveolin-1-expressing cells show partial inhibition of channel density at the surface while hSlo1Δ1 shows full inhibition? Several mechanisms could explain these findings. One can speculate that caveolin-1 association with Slo1 1007YNMLCFGIY1015 motif allosterically inhibits the export machinery association with the channel and that its deletion causes local structural protein rearrangements further enhancing this inhibition. Alternatively and assuming that, 1007YNMLCFGIY1015 forms part of the channel “export domain” one can hypothesize that in cells expressing hSlo1-WT + caveolin-1, caveolin-1, and export-machinery proteins compete for the same “pocket” containing 1007YNMLCFGIY1015; whereas in hSlo1Δ1, neither caveolin-1 nor the export machinery can associate with the channel resulting in lack of Slo1 surface expression. Because hSlo1 is a tetrameric channel, the degree of surface expression in the presence of caveolin-1 would vary depending on the number of occupied sites in hSlo1. In fact, a small fraction of cells transfected with hSlo1-WT + caveolin-1 showed both extremes, full or null surface expression. Thus, a delicate equilibrium of the hSlo1 empty occupied “export domain” shared between caveolin-1 and export-machinery proteins would define hSlo1 fate. Still a combination of allosteric and competition mechanisms may add to the complexity of caveolin-1 modulation of hSlo1 surface expression. Structural information on hSlo1 and caveolin-1 is needed to pinpoint exact mechanisms at the level of single residues.

The robust Slo1 expression at the surface of native vascular myocytes calls for different timings in Slo1 and caveolin-1 synthesis (both proteins can reach the plasma membrane when expressed independently). Conditions favoring the simultaneous synthesis of caveolin-1 with Slo1, as in our in vitro experiments, would prevent Slo1 surface expression conceivably to a degree depending on the saturation of caveolin-1 binding domains in Slo1. We recently showed that β1 subunit is able to increase endocytosis of hSlo1 in HEK293T cells reducing hSlo1 surface expression by −20% (40). Interestingly, caveolin-1-induced reduction of hSlo1 surface expression was not further enhanced by β1 (Fig. 6H) nor the degree of association between hSlo1 and caveolin-1 was affected by the regulatory subunit (Fig. 3). Taken together the results suggest that, in addition to retaining hSlo1 in intracellular compartments, once at the plasma membrane, caveolin-1 may stabilize hSlo1 as well.

Summarizing, the interaction of caveolin-1 with Slo1 may serve at least two non-exclusive roles in native tissues: (a) keeping part of Slo1 in caveolin-rich structures (caveolae) and (b) regulating Slo1 surface expression. One mechanism mediating these processes is likely the caveolin-1-binding motif of Slo1.

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