Increased Rho activation and PKC-mediated smooth muscle contractility in the absence of caveolin-1

Yulia Shakirova, Johan Bonnevier, Sebastian Albinsson, Mikael Adner, Bengt Rippe, Jonas Broman, Anders Arner, and Karl Sward

Department of Experimental Medical Science, Lund University, Department of Otorhinolaryngology, Malmö University Hospital, and Department of Physiology and Pharmacology, Karolinska Institute, Stockholm

Submitted 1 February 2006; accepted in final form 18 July 2006

Shakirova, Yulia, Johan Bonnevier, Sebastian Albinsson, Mikael Adner, Bengt Rippe, Jonas Broman, Anders Arner, and Karl Sward. Increased Rho activation and PKC-mediated smooth muscle contractility in the absence of caveolin-1. Am J Physiol Cell Physiol 291: C1326–C1335, 2006; doi:10.1152/ajpcell.00046.2006.—Caveolae are omega-shaped membrane invaginations that are abundant in smooth muscle cells. Since many receptors and signaling proteins co-localize with caveolae, these have been proposed to integrate important signaling pathways. The aim of this study was to test whether RhoA/Rho-kinase and protein kinase C (PKC)-mediated Ca\textsuperscript{2+} sensitization depends on caveolin using caveolin (Cav)-1-deficient (KO) and wild-type (WT) mice. In WT smooth muscle, caveolae were detected and Cav-1, -2, and -3 proteins were expressed. Relative mRNA expression levels were ~15:1:1 for Cav-1, -2, and -3, respectively. Caveolae were absent in KO and reduced levels of Cav-2 and Cav-3 proteins were seen. In intact ileum longitudinal muscle, no differences in the responses to 5-HT or the muscarinic agonist carbachol were found, whereas contraction elicited by endothelin-1 was reduced. Rho activation by GTP\textsubscript{yS} was increased in KO compared with WT as shown using a pull-down assay. Following α-toxin permeabilization, no difference in Ca\textsuperscript{2+} sensitivity or in Ca\textsuperscript{2+} sensitization was detected. In KO femoral arteries, phorbol 12,13-dibutyrate (PDBu)-induced and PKC-mediated contraction was increased. This was associated with increased α\textsubscript{1-adrenergic contraction. Following inhibition of PKC, α\textsubscript{1-adrenergic contraction was normalized. PDBu-induced Ca\textsuperscript{2+} sensitization was not increased in permeabilized femoral arteries. In conclusion, Rho activation, but not Ca\textsuperscript{2+} sensitization, depends on caveolae in the ileum. Moreover, PKC driven arterial contraction is increased in the absence of caveolin-1. This depends on an intact plasma membrane and is not associated with altered Ca\textsuperscript{2+} sensitivity.

Ca\textsuperscript{2+} sensitization; Rho-associated kinase; myosin phosphatase target protein; lipid rafts; CPI-17; G protein-coupled receptor

Caveolae are 50–100 nm flask-shaped membrane invaginations that are abundant in endothelial cells, adipocytes, and smooth muscle cells. Caveolae are characterized by high cholesterol and sphingolipid content, and a light buoyant density. They are stabilized by the caveolin proteins (9). Specific G protein-coupled and tyrosine kinase receptors, as well as downstream signaling intermediaries, have been shown to be caveolae-associated (26, 30). Such clustering has been envisioned to facilitate receptor signaling and has been proposed to play a role in receptor internalization. The scaffolding domain of caveolin-1 (Cav-1) may also function as a broad-spectrum kinase inhibitor (9).

Compared with endothelial and adipocyte caveolae, smooth muscle caveolae have received relatively little attention. With the use of cholesterol depletion to disrupt caveolae in denuded caudal arteries from the rat, we demonstrated that serotonin (5-HT\textsubscript{2A}) as well as endothelin-1 (ET-1) receptor signaling was impaired by cholesterol depletion. Moreover, restoration of caveolae by cholesterol replenishment recovered signaling from both receptors (3, 11). Cholesterol depletion was also shown to affect phasic but not tonic contraction in ureteric muscle (2), suggesting that raft/caveolae associated signaling influences specific steps in excitation-contraction coupling. Impaired contractile responses to ET-1, ANG II, and phorbol ester were found in arteries from Cav-1-deficient (KO) mice (10). The vessels did however, have intact endothelium, and the reduced contractility was interpreted to be secondary to enhanced nitric oxide synthase activity (29). In the urinary bladder, a general reduction of force on receptor stimulation was reported (37).

Downstream of receptor activation, RhoA, as well as Rho-kinase and protein kinase C (PKC) play key roles in regulation of contractility in smooth muscle (32). RhoA (15, 22, 34) and PKC (22, 32) have been shown to reside in or to translocate to caveolae on receptor stimulation. The scaffolding domain of Cav-1 was reported to impair both membrane translocation of PKC (34) and contraction stimulated by phorbol ester and α-agonist in the ferret aorta (16). Further support for a role of caveolae in PKC signaling is the finding that phosphoinositide turnover is compartmentalized in this membrane microdomain as shown using biochemical fractionation (27). Finally, Rho-kinase has been demonstrated to translocate to caveolae in a Ca\textsuperscript{2+}-calmodulin-dependent manner on smooth muscle depolarization (36). Taken together, these results suggest that caveolae may play a role in contractility and in regulation of Ca\textsuperscript{2+} sensitivity of contraction in smooth muscle (4).

In the present study, using KO mice, we investigate whether PKC and RhoA/Rho-kinase-mediated contractile mechanisms depend on caveolae. We have used longitudinal smooth muscle from the small intestine, which shows robust RhoA/Rho-kinase-mediated Ca\textsuperscript{2+} sensitization, but weak sensitization in response to PKC activation with phorbol ester (8, 21, 33), and femoral artery, which displays prominent Ca\textsuperscript{2+} sensitization in response to phorbol ester (8, 38).

Materials and Methods

KO mice. Male KO mice were obtained from the Jackson Laboratory (Bar Harbor, ME), back-crossed six times onto the C57BL/6 background.
background, and genotyped as described by Razani et al. (28). Age-matched (10–15 wk) male C57BL/6 (Møllegård, Copenhagen, Denmark) were used as controls and are referred to as wild type (WT). Weights of KO and WT mice were 27.4 ± 0.9 and 27.3 ± 0.7 g, respectively. Mice were anesthetized with isoflurane and euthanized by cervical dislocation. The experiments were performed according to European guidelines for animal research and approved by the local animal ethics committee.

Preparation of intestine and femoral artery. The intestine and femoral artery were removed and placed in cold HEPS-buffered physiological saline solution (for composition, see below). Strips from the outer longitudinal small intestinal muscle layer were obtained by tearing the longitudinal layer off from the underlying circular layer (8).

Electron microscopy. Preparations were fixed in 2% glutaraldehyde and 1% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4, 300 mosM), postfixed in OsO4, dehydrated, and embedded in Durcupan ACM (Fluka, Buchs, Switzerland). Ultrathin sections were examined with the use of transmission electron microscopy (3, 11).

Western blot analysis. Tissue preparations were frozen using clamps precooled in liquid N2. Frozen tissue was pulverized and 300 mosM), postfixed in OsO4, dehydrated, and embedded in Durcupan ACM (Fluka, Buchs, Switzerland). Ultra thin sections were examined with the use of transmission electron microscopy (3, 11).

Immunofluorescence staining of Cav-1 and -3. Tubular ileum segments were pulled over 20-μl plastic pipette tips, which were immersed in Histochocie (Amresco) for at least 4 h. After incubation in fixative, tissues were thoroughly washed in 70% ethanol and maintained therein at 4°C for at least 3 days, with three solution changes, until further processing. Following incubation in 96% (2 h) and 100% ethanol (1 h), 1:1 ethanol:xylene (30 min) and xylene (1 h), tissues were immersed in paraffin (2 × 1 h) and embedded. Sections measuring 10 μm were cut and deparaffinized. The sections were washed in DNA dye Sytox Green (1:3,000, Molecular Probes) after a brief washing in PBS. Pictures were obtained in a Zeiss LSM 510 confocal microscope. Caveolin proteins were detected by monitoring Cy5 fluorescence upon excitation at 633 nm. Sytox Green fluorescence was monitored upon excitation at 488 nm. Primary and secondary antibody omission controls verified specificity of staining (Fig. 3).

RhoA activation assay. RhoA activation was assayed using a kit from Upstate Biotechnology. Longitudinal ileum strips were homogenized in ice-cold lysis buffer containing 25 mM HEPS (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1% Igepal CA-630, and 10% glycerol. After centrifugation at 14,000 g for 15 min, a small portion of the supernatant was removed for protein determination. The remaining supernatant was divided in two equal aliquots and 100 μM GTPγS was added to one of them. Activated RhoA was precipitated using the Rho-binding domain from Rhotekin coupled via GST to GSH beads. Loads and precipitates were analyzed by Western blotting using the antibody against Rho (A-C) supplied with the kit, or the Cav-1 and ET-1 antibodies described above.
preparations weighed too little (~60 μg) to weigh reliably with standard equipment. We therefore recorded force and length from four preparations and weighed them pooled together for one stress determination. Wet weight was obtained using a Mettler M3 balance after gentle blotting of the tissue on a filter paper.

**Force recording, α-toxin-permeabilized preparations.** Intestinal strips, 4-mm long, 1-mm wide, and 50-μm thick, were wrapped at both ends with aluminum foil. Femoral artery segments (2 mm long, cut as spiral strips from the media) were mounted on two 31 m steel wires (31). All preparations were mounted horizontally between two tungsten wires, one of which was attached to a force transducer (model AE 801, SensoNor, Horten, Norway) and the other to a micrometer screw (8). Experiments were performed on “bubble plates” (140 l solution) with stirring. Preparations were equilibrated in HEPES-buffered physiological saline solution and a high-K⁺ (80 mM) test contraction was induced. Following relaxation, preparations were permeabilized with *Staphylococcus aureus* α-toxin (10,000 U/ml, Calbiochem-EMD Biosciences, San Diego, CA) for 60 min. Permeabilized preparations were treated with 10 μM A23187 for 20 min to deplete intracellular Ca²⁺ stores. Experiments were run at room temperature (22°C).

**Solutions.** The HEPES-buffered physiological saline solution for dissection contained (in mM) 135.5 NaCl, 5.9 KCl, 1.2 MgCl₂, 11.6 glucose, and 11.6 HEPES, pH 7.4. The Krebs solution, for experiments on intact small intestinal muscle tissue, contained (in mM) 122 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, and 11.5 glucose. For permeabilized preparations, the relaxation solution (pCa 9) contained (in mM) 30 N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 10 phosphocreatine, 5.14 Na₂ATP, 7.92 Mg-acetate, 46.6 K⁺-methanesulfonate, 10 EGTA, and 1 DTE. The contraction solution (pCa 4.5) was made by replacing EGTA with Ca-EGTA. Ionic strength (0.15 M) and free [Mg²⁺] (2 mM) were held constant by adjusting [K⁺]-methanesulfonate and [Mg-acetate], pH was adjusted to 7.1. Fixed free Ca²⁺ concentrations were obtained by mixing relaxation and contraction solutions.

**Drugs.** Y-27632 and microcystin-LR was obtained from Calbiochem-EMD Biosciences. All other drugs used were obtained from Sigma.

**Statistics.** Values are means ± SE. Unless stated otherwise, n refers to the number of mice. Statistical significance was determined using Student’s t-test for unpaired data.

**RESULTS**

**Caveolae.** Caveolae were seen by electron microscopy in longitudinal smooth muscle from the WT mouse ileum (Fig. 1, top left, note arrowheads) and femoral artery (Fig. 1, bottom left). Caveolae were not found in longitudinal smooth muscle from KO mice (Fig. 1, top right). No caveolae were found in femoral artery smooth muscle cells from KO (Fig. 1, bottom right).

**Caveolin detection by Western blot analysis.** Cav-1 (α and β), Cav-2 (α and β), and Cav-3 were detected by Western blot analysis in WT longitudinal smooth muscle from the ileum (Fig. 2A). Cav-1 and Cav-2 were not detected in KO ileum (Fig. 2A). Reduced Cav-2 content in Cav-1/−/− tissues has been reported previously (10, 28). In the KO ileum, expression of Cav-3 was also reduced (by 76 ± 6% vs. WT, n = 7, P < 0.001; Fig. 2A). Cav-1, Cav-2, and Cav-3 were absent, or exhibited considerably reduced expression, in the denuded femoral artery from KO (Fig. 2B). To verify reduced expression of Cav-3, which was unexpected, Cav-3 expression in the ileum was normalized to β-actin (Fig. 2C). This confirmed reduced contents in relation to a housekeeping protein in KO relative to WT (n = 4, P < 0.05). Dilutions of extracts, blotted
for Cav-3, also verified reduced Cav-3 expression in the longitudinal smooth muscle from ileum (Fig. 2D).

**Immunofluorescence staining of Cav-1 and Cav-3 in ileum.**
Cav-1 and Cav-3 were visualized by immunofluorescence. Cav-1 staining revealed 7–15 clusters per cell membrane profile (red in Fig. 3, *top left*) in ileum longitudinal smooth muscle. Cav-3 staining showed some degree of clustering along cell membrane profiles in WT muscle (Fig. 3). In KO, Cav-3 clustering was lost and staining appeared inside cells (Fig. 3).

---

**Fig. 3.** Immunofluorescence staining of Cav-1 and Cav-3 (red) and nuclei (green) in paraffin embedded longitudinal ileum muscle from WT and KO mice. Visceral peritoneum is oriented upward and the longitudinal smooth muscle cells are cross-sectioned. *Top left:* caveolin-1 staining in WT. *Left middle and bottom:* omission controls. *Top right:* caveolin-3 staining in WT. *Right bottom:* Cav-3 staining in KO mice. Scale bars = 10 μm.

---

**Fig. 2.** *A:* Western blots of samples from WT and KO longitudinal smooth muscle from ileum, using antibodies against Cav-1, -2, and -3. Identical amounts of protein (20 μg) were loaded in all lanes (*n* = 7). *B:* blots using femoral artery homogenates (*n* = 4). *C:* to confirm reduced Cav-3 expression in the longitudinal smooth muscle from ileum and verify equal loading, Cav-3 and β-actin antibodies were used together (*n* = 4, *P* < 0.05 for Cav-3/β-actin ratio). Numbers above bands indicate optical density × mm² for the respective bands. *D:* regression lines of optical density × mm² for Cav-3 vs. micrograms of loaded protein in WT (○) and KO (●). Slopes were significantly reduced in KO vs. WT (*P* < 0.05, *n* = 4) verifying reduced Cav-3 in Cav-1-deficient smooth muscle.
Quantitative real-time PCR. In WT longitudinal muscle Cav-1 mRNA levels were ~15-fold higher than those of Cav-2 and Cav-3 (Fig. 4). No differences in mRNA between WT and KO tissues were obtained for Cav-2. The mRNA levels for Cav-3 were lower in KO compared with WT longitudinal smooth muscle preparations but this difference did not reach significance \((P = 0.091)\). The tissues used for PCR (Fig. 4) correspond with that used for blotting in Fig. 2A.

Contractile properties of intact longitudinal smooth muscle from the ileum. High-K\(^+\) induced contractions were similar in WT and KO ileum longitudinal muscle (WT: \(8.6 \pm 0.4\) KO \(8.1 \pm 0.9\) mN, Krebs solution, \(37^\circ C\), \(n = 12\)). Since WT and KO preparations were of similar sizes, these results suggest that active force generation in response to membrane depolarization is unchanged in KO.

Force responses to receptor agonists were examined in intact ileum strips. Original records are shown in Fig. 5A. Amplitudes of the carbachol and 5-HT responses were unchanged in the KO preparations (Fig. 5B). The EC\(_{50}\) values for carbachol in KO and WT ileum were not different (WT: \(0.35 \pm 0.08\); KO: \(0.34 \pm 0.03\) \(\mu\)M, \(n = 8\)), and contractions exhibited a rapid phase followed by sustained contraction in both KO and WT. Contractions in response to 10 nM ET-1 were reduced by 50% in small intestinal tissue from KO compared with WT mice (Fig. 5).

Rho activation in longitudinal smooth muscle from the ileum. RhoA plays an important role in the sustained phase of muscarinic contractin the ileum (21). To assess activation of Rho in WT and KO ileum, a pull-down assay exploiting the specific GTP dependent association of Rho with the Rho-binding domain in Rhotekin coupled via GST to glutathione beads, was used (Fig. 6). Homogenates and precipitates were blotted for Rho, Cav-1, and ET-1 receptor A (ETA). Rho was activated by GTP\(_\gamma\)S (Fig. 6A). Basal Rho-activity was below the detection limit (Fig. 6B). The Rho-activation level was increased in KO vs. WT muscle after incubation with GTP\(_\gamma\)S (\(n = 5, P < 0.05\)). Moreover, Cav-1 was precipitated together with Rho in WT but not in KO. Some ETA receptor also coprecipitated with Rho. The amount of Rho (A, B, C) and ETA was similar in WT and KO homogenates (Fig. 6A, left lanes). In six experiments on independent homogenates, ETA receptor expression was 114 \(\pm\) 13% in KO relative to WT (\(P > 0.05\)).

Force responses of \(\alpha\)-toxin permeabilized longitudinal smooth muscle from the ileum. To examine whether \(Ca^{2+}\) sensitization might be affected in the absence of caveolae, we used \(\alpha\)-toxin permeabilized smooth muscle. In the ileum, Rho/Rho-kinase mediated modulation of \(Ca^{2+}\) sensitivity is prominent and well characterized (8, 18, 33). The pCa-force relationships were identical in \(\alpha\)-toxin permeabilized strips from WT and KO (Fig. 7A). Following a submaximal \(Ca^{2+}\) concentration (pCa 6.0), strips were stimulated with carbachol (10 \(\mu\)M; in the presence of 30 \(\mu\)M GTP) and GTP\(_\gamma\)S (10 \(\mu\)M). A maximal contraction was finally achieved by inhibiting the myosin phosphatase with 1 \(\mu\)M microcystin-LR. \(Ca^{2+}\) sensitization was not affected in KO compared with WT, irrespective of stimulus (Fig. 7B). Separate experiments showed that ET-1 (10 nM following 30 \(\mu\)M GTP) induced little or no \(Ca^{2+}\) sensitization in the mouse ileum (not shown).

To address the possibility that dependence of \(Ca^{2+}\) sensitization on Rho-kinase might be different in KO vs. WT, 30 \(\mu\)M of specific Rho-kinase inhibitor Y27632 was used. Y27632

![Fig. 4. Real-time PCR to assess the mRNA levels for Cav-1, -2, and -3 in dissected longitudinal smooth muscle from the mouse small intestine from WT and KO mice. mRNA is expressed in relation to the internal control gene \(\beta\)-actin as \(2^{\Delta\Delta\text{Ct}} \times 10^3\) and presented as means \(\pm\) SE (\(n = 7–8\)). Ct, cycle threshold.](http://ajpcell.physiology.org/issue/)

![Fig. 5. A: original traces of force in longitudinal ileum muscle preparations from WT and KO animals. The preparations were activated with high-K\(^+\) (80 mM KCl), 10 \(\mu\)M carbachol (CCh), and 10 nM endothelin-1 (ET-1). ET-1 gave rise to phasic contractions superimposed on a sustained contraction. B: mean values of the maximal force responses to 10 \(\mu\)M CCh, 1 \(\mu\)M 5-HT, and 10 nM ET-1 in intact longitudinal smooth muscle from the ileum of WT (open bars) and KO (solid bars) mice. Force is expressed relative to the peak of the high-K\(^+\) responses. \(*P < 0.05, n = 8.\) (C) force responses to 10 nM ET-1 at 5 min.](http://ajpcell.physiology.org/issue/)
inhibited GTPγS-induced contraction completely in both WT and KO mice (not shown).

**PKC-mediated contraction in intact femoral arteries.** Since PKC responses have been shown to depend on CPI-17 (19, 38), and the CPI-17 content is low in longitudinal smooth muscle from the ileum, we investigated whether PKC-mediated contraction was different using femoral arteries. In the femoral artery, the CPI-17 content is high and phorbol 12,13-dibutyrate (PDBu), which activates PKC, gives rise to a sizeable contraction (8). Experiments on denuded, but otherwise intact, ring preparations of the femoral artery were made in the continuous presence of 300 μM L-NAME to avoid residual nitric oxide synthesis. Following relaxation from contraction induced by 80 mM K⁺ (HK), 1 μM PDBu was added (Fig. 8A). The time course of contraction in response to PDBu was slower in KO, but the level of force reached after 30 min was increased (Fig. 8, A and B, n = 16 WT and 16 KO preparations from 4 WT/KO pairs of mice, P < 0.05). After 30 min in the PDBu solution, the PKC inhibitor GF 109203X (GFX) was added in a cumulative manner (Fig. 8, A and C). The IC₅₀ value for GFX was not different in KO vs. WT (0.31 ± 0.05 μM for WT and 0.40 ± 0.07 μM for KO, P > 0.05). Expression of PKC-α, as assessed by Western blot analysis, was not different in KO (KO: 106 ± 12% of WT, n = 8).

α₁-Adrenergic responses in rabbit femoral artery are mediated in part by PKC (17). We compared the concentration response relations for the selective α₁-adrenergic receptor agonist cirazoline in WT and KO femoral arteries in the presence of L-NAME. The typical pattern was sustained contraction with little inactivation, such as that shown in Fig. 9A. Phasic repetitive activity sometimes occurred, and contraction often inactivated faster in WT than in KO in those cases. Force was averaged during the time that each concentration was maintained. Consistent with increased PKC-mediated contraction in KO, cirazoline responses were increased, both when expressed as absolute force (Fig. 9B), and following normalization to contraction induced by 80 mM K⁺ (Fig. 9C), which was unchanged (bars in Fig. 9B). EC₅₀ values for cirazoline were not different (0.17 ± 0.08 in KO vs. 0.36 ± 0.19 μM in WT, n = 16 preparations from 4 animals of each genotype; P > 0.05).

To measure stress, the length between attachments and the wet weight of the femoral artery preparations were determined. Wet weight per millimeter of tube length of femoral artery was greater in KO than in WT (42 ± 5 vs. 34 ± 4 μg/mm, n = 8).
10 mice of each genotype; $P < 0.001$). Consequently, stress was lower in response to depolarization in KO compared with WT, whereas the cirazoline-induced stress was unchanged (Fig. 9D).

$\alpha_1$-Adrenergic contraction was next examined in the presence of the PKC inhibitor GFX. Cirazoline-induced force was not different following PKC inhibition ($n = 8$ preparations from 2 WT/KO pairs; Fig. 9E). Vehicle-treated preparations from the same animals confirmed the difference in cirazoline-induced force between WT and KO.

PKC mediated contraction in permeabilized femoral arteries. To address whether increased PDBu-induced and PKC-mediated contraction was due to increased $\mathrm{Ca}^{2+}$ sensitization, $\alpha$-toxin-permeabilized spiral strips from the femoral artery

---

**Fig. 8.** A: original records of contraction in response to phorbol ester in intact femoral arteries from WT and KO. Following depolarization with 80 mM K$^+$ (HK), arteries were incubated with phorbol 12, 13-dibutyrate (PDBu; 1 $\mu$M) for 30 min. The protein kinase C inhibitor GF 109203X was subsequently added in a cumulative manner. Horizontal and vertical calibration bars represent 1 mN/mm and 5 min, respectively. 300 $\mu$M $N^\omega$-nitro-$l$-arginine methyl ester ($l$-NAME) was present throughout. B: summarized force data, expressed as mN/mm ($left$), and as a percentage of HK ($right$) for WT (open bars) and KO (solid bars). C: compiled data from the concentration response experiments with GF 109203X (WT, $\odot$; KO, $\bullet$). $n = 16$ and 16 preparations, respectively, from 4 WT/KO pairs.

---

**Fig. 9.** A: concentration response relationships for the $\alpha_1$ adrenergic receptor agonist cirazoline (Cir) in intact femoral arteries from WT and KO mice. Horizontal and vertical calibration bars represent 1 mN/mm and 5 min, respectively. 300 $\mu$M $l$-NAME was present throughout. B: summarized force data, expressed as mN/mm, during the concentration response experiments depicted in A. Bars in B show force in response to 80 mM K$^+$ in WT (open) and KO (solid). C: force in WT ($\odot$) and KO ($\bullet$) expressed instead as a percentage of 80 mM K$^+$ contraction. $n = 16$ and 16 preparations, respectively, from 4 WT/KO pairs. D: force expressed as stress. Cross-sectional area was obtained from the weight and distance between attachments. $n = 40$ preparations pooled in 10 groups from 10 mice of each genotype. E: concentration response relations for cirazoline in the presence ($\odot$) and absence ($\bullet$) of the protein kinase C inhibitor GF 109203X (5 $\mu$M). $n = 8$ preparations from 2 mice of each genotype for both treatments. $*P < 0.05$ for KO vs. WT comparison.
were used. Force was normalized to a reference (pCa 4.5) contraction. The response to an intermediate Ca\(^{2+}\) concentration (pCa = 6.5) was similar in strips from WT and KO mice. Moreover, the addition of increasing amounts of PDBu caused Ca\(^{2+}\) sensitization that was identical in WT and KO (Fig. 10, A and B). Results were the same when data was normalized to depolarization-induced contraction obtained before permeabilization (not shown). Phosphorylation of CPI-17 was similar in intact preparations from WT and KO after 30-min incubation with 1 \(\mu\)M PDBu (WT: 100 ± 9%, KO: 102 ± 10%, \(n = 4\)).

**DISCUSSION**

In this study, we show that Rho activation and PKC mediated contraction of smooth muscle is increased in the absence of caveolae and Cav-1. Ablation of caveolae attenuated endothelin-induced contraction in the ileum, without influencing muscarinic or serotonergic force. Increased Rho activation and PKC-mediated contraction may occur in other cell types, including endothelial cells and fibroblasts.

All caveolin family members were expressed in smooth muscle. In agreement with previous studies (10, 28, 37), ablation of Cav-1 was associated with reduction of the Cav-2 protein(s). We also made the unexpected observation that the Cav-3 protein content was reduced and its distribution altered in KO mice. The relative mRNA abundance for Cav-1, -2, and -3 was found to be 15:1:1 in the ileum. This suggests that an absolute minority (7%) of caveolae in WT intestinal smooth muscle are Cav-3 driven. The relative levels of expression, the intracellular Cav-3 accumulation, and the observed reduction of Cav-3 protein, probably all explain the apparent absence of caveolae in electron micrographs from KO tissue. The basis of the reduced Cav-3 protein expression is unclear. Cav-3 mRNA levels were not different, which suggests a mechanism involving Cav-3 protein degradation. Importantly, however, the apparent absence of caveolae and the reduced membrane association of Cav-3 in the KO argue against the possibility that the remaining Cav-3 compensates for the lack of Cav-1 in smooth muscle.

Caveolae have been suggested to play a role in signaling from smooth muscle ET-1 and 5-HT\(_{2A}\) receptors (3, 6, 11). M\(_2\) muscarinic receptors have been shown to translocate to caveolae on agonist binding which was suggested to be important for downstream signaling (12). Finally, caveolae have been proposed to play a role in M\(_2\) and M\(_3\) receptor desensitization (25). In support of a role of caveolae in muscarinic signaling, contraction was found to be selectively impaired (by 70%) in the bladder of KO mice (20), although another study suggested a reduction of contraction elicited by both carbachol and KCl in the bladder (37). We find that contractions in response to 5-HT and the muscarinic agonist carbachol are unchanged in intestinal muscle lacking caveolae when expressed relative to a reference high-K\(^+\) contraction. Serotonergic contractions of longitudinal smooth muscle from the small intestine have been reported to be due to 5-HT\(_1\) and 5-HT\(_3\) receptors (39). The contribution to contraction of 5-HT\(_{2A}\) receptors, which may be caveolae associated (6, 11, 13), could thus be small in the ileum longitudinal smooth muscle.

It may be relevant in regard to endothelin-induced contraction that both ET\(_A\) and ET\(_B\) receptors have been proposed to depend on caveolae (3, 35). The present results showed that impaired contractility in response to ET-1 in the ileum was not associated with reduced ET-1 receptor expression, nor was it due to reduced Ca\(^{2+}\) sensitization. Further studies of endothelin signaling in the absence of caveolae seem warranted.

On the basis of the reported translocation to caveolae of key components of Ca\(^{2+}\) sensitization, we formulated the hypothesis that Ca\(^{2+}\) sensitization would be affected in the absence of caveolae. Our results confirm the association between Rho and Cav-1 that was previously observed in endothelial cells and fibroblasts (15, 22). This interaction appears to be functionally inhibitory since Rho activation was greater in KO. Our data on permeabilized ileum suggest, however, that Ca\(^{2+}\)-sensitization proceeds normally in permeabilized muscle without caveolae and Cav-1. It therefore has to be assumed that GTP\(_y\)S-induced Ca\(^{2+}\) sensitization is not limited by Rho activation in the mouse ileum. Alternatively, Rho activation by ligand plus GTP may not be changed. We also failed to detect a difference in the sustained phase of carbachol contraction, which is known to depend on RhoA/Rho-kinase (21, 33). This indicates that Ca\(^{2+}\)-sensitization is unchanged in situ in the absence of caveolae. A modest change in stress (force per cross-sectional area) cannot be ruled out, nor can it be ruled out that Ca\(^{2+}\)-sensitization mechanisms have adapted by a fine tuning of the expression of downstream intermediaries, but caveolae and Cav-1 are clearly

---

**Fig. 10.** Ca\(^{2+}\) sensitivity and Ca\(^{2+}\) sensitization of force in femoral arteries. A: \(\alpha\)-toxin permeabilized femoral artery strips from WT (open bars) and KO (solid bars) at intermediate [Ca\(^{2+}\)] (pCa 6.5), activated with PDBu (1 \(\mu\)M) or the phosphatase inhibitor microcystin-LR (1 \(\mu\)M). Force is expressed relative to a reference (pCa 4.5) contraction. B: force at increasing PDBu concentrations in femoral arteries activated at pCa 6.5, \(n = 4\) preparations from 4 mice of each genotype.
not required for a functional pathway, albeit subtly affecting maximal RhoA activation.

PKC-mediated contraction in the intact femoral artery was increased in the KO relative to the WT. PKC protein expression was not changed as shown directly for PKC-α, and as suggested by the Ca^{2+}-sensitization study. The data is thus compatible with the removal of an inhibitory influence of Cav-1 similar to the situation with Rho. Moreover, α1-adrenergic contraction was increased as expected for a response mediated partly by PKC. α1-Adrenergic contraction was not enhanced following inhibition of PKC; thus creating a strong argument that enhanced PKC signaling in KO compared with WT indeed underlies the increased α1-adrenergic contraction. The unchanged Ca^{2+} sensitization following permeabilization, and the unchanged phosphorylation of CPI-17, demonstrate that the increased PKC-induced contraction is due to a membrane-delimited mechanism. It seems reasonable to propose that Cav-1 is effective as a kinase inhibitor only in the sarcolemma, where it is located, and not intracellularly, where substrates such as CPI-17 are found. A variety of membrane-associated effector mechanisms, which could mediate the PKC effect, have been described. Examples include the delayed rectifier current (1), K_{ATP} channels (5), and Ca^{2+} sparks (7), all of which are bypassed in the α-toxin permeabilized preparations. It is notable that the frequency of spontaneous transient outward currents, which are activated by Ca^{2+} sparks, are reduced in cerebral arterial myocytes from Cav-1-deficient mice (10). Whether this reduction is normalized by PKC inhibition is not known.

The possible association of α1-adrenergic receptors with caveolae is controversial. We previously examined the distribution of α1A-receptors in sucrose density gradients of smooth muscle homogenates from the rat caudal artery, and found that the α1A-receptors were present in the fractions of highest density, contrasting with 5HT_{2A} receptors which were present in the lighter caveolin-containing fractions (11). On the other hand, binding of isotope-labeled phenylephrin and prazosin to caveolin-containing sucrose gradient fractions from heart and aorta was observed by others (14, 24). The latter studies support a caveolar association of α1-receptors in those specific cells and tissues. The present data does not distinguish between the possible α1-receptor locales, which may be tissue and receptor subtype specific. Alleviated inhibition of PKC by caveolin at the membrane is sufficient to explain the present results in the femoral artery.

Measurements of stress (force per cross-sectional area), using traditional methodology involving weighing, showed that depolarization-induced stress was reduced in KO compared with WT femoral arteries. This was due to a significant increase in the wet weight per mm length of femoral artery rather than a change in depolarization induced force. A detailed morphometric analysis needs to be made to justify such a stress calculation, because the increased wet weight may be due to increased extracellular matrix or increased adventitial cell populations. Moreover, force per length of arterial tube, not stress, is the relevant variable for regulation of peripheral resistance. In permeabilized tissue it is notoriously difficult to minimize experimental variation in stress and absolute force determinations. This is because cutting of strips involves considerable handling that may harm the tissue, the small weight of the preparations (=60 μg), and because the success of permeabilization varies. We can therefore not rule out changes in stress or absolute force in the permeabilized strips. Our data in intact tissue justifies normalization to a reference contraction, however, and the unchanged phosphorylation of CPI-17 independently shows that PKC-driven Ca^{2+} sensitization is unaltered in the absence of caveolae.

Our results concerning PKC-mediated contraction are fully compatible with the inhibitory effect seen after chemical loading of the scaffolding domain peptide from Cav-1 in the intact ferret aorta (16).

In conclusion, PKC mediated contraction and Rho activation but not Ca^{2+}-sensitization are increased in smooth muscle following genetic ablation of Cav-1. Increased RhoA activation and PKC-driven force generation in the absence of caveolae may play a role in contractility or motility in many cell types, including, apart from smooth muscle cells, endothelial cells and fibroblasts.

ACKNOWLEDGMENTS

We thank Inggered Larsson for technical assistance in the mRNA study, Gunnel Roos for genotyping, Karin Janssner for advice regarding histology, and Prof. Rupert Hallmann for advice, support, and help with establishing the Cav-1-deficient mouse colony.

GRANTS

This study was supported by Swedish Research Council Grants 71X-14955 (to K. Swärd), 04X-8268 (to A. Arner), 04X-08285–17A (to B. Rippe), funds from the Crafoord, Åke Wiberg, and Magnus Bergvall foundations (to K. Swärd), the Royal Physiographic Society (K. Swärd), and the Swedish Heart-Lung foundation (to K. Swärd and A. Arner). This study was supported by a program grant from the Ragnar Söderberg Foundation to the Vascular Wall Program at Lund University.

REFERENCES


