Cholesterol Depletion Disrupts Caveolae and Differentially Impairs Agonist-Induced Arterial Contraction

Karl Dreja, Marianne Voldstedlund, Jørgen Vinten, Jørgen Tranum-Jensen, Per Hellstrand, Karl Swärd

Objective—This study assessed the role of cholesterol-rich membrane regions, including caveolae, in the regulation of arterial contractility.

Methods and Results—Rat tail artery devoid of endothelium was treated with the cholesterol acceptor methyl-β-cyclodextrin, and the effects on force and Ca²⁺ handling were evaluated. In cholesterol-depleted preparations, the force responses to α₁-adrenergic receptors, membrane depolarization, inhibition of myosin light chain phosphatase, and activation of G proteins with a mixture of 20 mmol/L NaF and 60 μmol/L AlCl₃ were unaffected. In contrast, responses to 5-hydroxytryptamine (5-HT), vasopressin, and endothelin were reduced by >50%. The rise in global intracellular free Ca²⁺ concentration in response to 5-HT was attenuated, as was the generation of Ca²⁺ waves at the cellular level. By electron microscopy, cholesterol depletion was found to disrupt caveolae. The 5-HT response could be restored by exogenous cholesterol, which also restored caveolae. Western blots showed that the levels of 5-HT₂A receptor and of caveolin-1 were unaffected by cholesterol extraction. Sucrose gradient centrifugation showed enrichment of 5-HT₂A receptors, but not α₁-adrenergic receptors, in the caveolin-1–containing fractions, suggesting localization of the former to caveolae.

Conclusions—These results show that a subset of signaling pathways that regulate smooth muscle contraction depends specifically on cholesterol. Furthermore, the cholesterol-dependent step in serotonergic signaling occurs early in the pathway and depends on the integrity of caveolae. (Arterioscler Thromb Vasc Biol. 2002;22:1267-1272.)

Key Words: smooth muscle • caveolae • 5-hydroxytryptamine • endothelin • intracellular calcium

Cellular cholesterol, of which most (up to 90%) resides in the plasma membrane, is crucial for normal membrane permeability and fluidity and also plays a role in cellular signaling, via several proposed mechanisms that fall into at least 4 categories. First, cellular cholesterol may influence gene transcription in the nucleus through sterol regulatory element binding proteins. Second, the activity of membrane receptors, ion channels, and transporters may depend on the membrane fluidity, per se. Third, membrane protein function may be regulated through specific cholesterol-protein interactions. Fourth, cholesterol stabilizes the structure of caveolae and lipid rafts.

Caveolae, which are 50- to 100-nm membrane invaginations that are abundant in vascular endothelium and smooth muscle cells, are defined by their characteristic morphology and contents of caveolin and cav-p60. No definitive definition of rafts has appeared because they do not exhibit a characteristic structure, but the term is used for planar aggregations of specific lipids and proteins. Caveolae and lipid rafts are envisaged to serve as platforms for a dynamic association of signaling proteins and for the initiation or modulation of signaling.

Some agonists causing contraction of vascular smooth muscle act on receptors that are believed to be located in caveolae (eg, endothelin A receptors) or to aggregate in caveolae on ligand binding (eg, angiotensin type 1 or bradykinin B₂ receptors). Cholesterol-rich membrane domains may be considered as possible physical platforms for the coding of intracellular signals. To investigate this, intracellular Ca²⁺ and contractile responses to agonist stimulation were studied in arterial muscle after depletion of cholesterol by methyl-β-cyclodextrin (mΒcd). This disrupts caveolar structure, whereas the general morphology of the tissue is preserved. Because the present study was performed on whole tissue, the contractile phenotype and cell-surface contacts were preserved; thus, contractile responses and normal ion homeostasis were maintained. The results suggest that although cholesterol concentration and integrity of caveolae are not critical for contraction or responses to α₁-adrenergic agonists, they profoundly influence a specific set of signaling pathways, including those for serotonin, vasopressin, and endothelin.
Methods
Preparation of the Tail Artery
Female Sprague-Dawley rats were euthanized by cervical dislocation, as approved by the regional ethics committee. The tail artery was dissected free in nominally Ca\(^{2+}\)-free HEPES-buffered Krebs solution (135.5 mmol/L NaCl, 5.9 mmol/L KCl, 1.2 mmol/L MgCl\(_2\), 11.6 mmol/L glucose, and 11.6 mmol/L HEPES, pH 7.35) and slid over an insect needle to remove the endothelium. Endothelial denudation by this procedure was verified by confocal and electron microscopy. Additionally, in toluidine blue-stained paraffin sections examined by light microscopy, the endothelium appeared to be absent (data not shown).

Force Measurements
Ring segments (1 mm) were mounted for force registration and equilibrated as described. Preparations were contracted with agonist for 7 minutes, interspersed by 25-minute relaxation periods, until stable contractions were attained. They were then treated with 10 mmol/L mβcd for 1 hour at 37°C. Extraction was temperature sensitive, and incubations at room temperature were completely ineffective. Longer treatment (3 hours) at 37°C reduced high K\(^+\) responses and appeared to affect membrane integrity, as judged by increased leakage of fura 2 and enhanced basal Mn\(^{2+}\) quench rate (data not shown). Additionally, all caveolae had disappeared, and in approximately one third of the cell profiles, signs of cellular degeneration had appeared, including swollen mitochondria and membrane-bound inclusions in the cytoplasm and the nucleus.

Fixation and Microscopy
For electron microscopy, arterial segments were fixed in sodium cacodylate (100 mmol/L), sucrose (170 mmol/L), CaCl\(_2\) (1.5 mmol/L), and glutaraldehyde (2%), with pH adjusted to 7.4. After 1 to 2 hours, the preparations were transferred to the same solution devoid of glutaraldehyde and stored at 4°C. The preparations were transferred to the same solution devoid of glutaraldehyde and stored at 4°C. The sections were contrasted by lead citrate and bismuth subnitrate. Images were obtained with a Philips 210 transmission electron microscope.

Segments for immunofluorescence microscopy were fixed in 96% ethanol, embedded in paraffin, and sectioned for indirect immunofluorescence. Control sections omitting the primary antibody were devoid of specific fluorescence signal.

[Ca\(^{2+}\)]\(_i\) Measurements
Segments (2 mm) of tail artery were mounted and incubated with fura 2-AM as described. They were then incubated for 1 hour with or without 10 mmol/L mβcd at 37°C in HEPES-buffered Krebs solution. The intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was measured by epifluorescence at 37°C. For subcellular [Ca\(^{2+}\)]\(_i\), measurements, segments were incubated with fluo 4-AM for 90 minutes after exposure to mβcd or control solution for 1 hour. Ca\(^{2+}\) wave activity was measured using a Zeiss 510 laser scanning confocal microscope.

Western Blotting
Proteins were extracted and separated on 12% SDS-PAGE gels, and Western blotting on nitrocellulose membranes was then performed essentially as described, with the use of antibodies to α\(_{1A}\)-adrenergic receptor (1:50), caveolin-1 (68E, 1 µg/mL), 5-hydroxytryptamine (5-HT)\(_{3A}\) receptor (1:500), or Cav-60 (1 µg/mL). Peroxidase-conjugated secondary antibodies were used, and peroxidase activity was detected by chemiluminescence (Pierce). Preparative of Caveolin-Enriched Fractions
Three to 5 freshly dissected tail arteries were transferred to 1 mL of 500 mmol/L sodium carbonate, at pH 11.0, cut manually, homogenized with a Polytron (Janke & Kunkel) tissue grinder (three 20-second bursts), and sonicated (three 20-second bursts). Five percent to 45% discontinuous sucrose gradient centrifugation was performed as described by Song et al. Ten fractions were recovered, and the 5%-35% interface was expected in fraction 5, which also contained the majority of caveolin-1.

Chemicals and Reagents
The primary antibodies 6B6 and 2F11 are protein-G–purified mouse monoclonal antibodies (Mabs) against caveolin-1 and caveolin-2. Anti–caveolin-1 polyclonal antibody (catalog No. c13630), anti–caveolin-2 Mab (clone 65, catalog No. C57820), and anti–caveolin-3 Mab (clone 26, catalog No. C38320) were all from Transduction Laboratories. FITC-conjugated secondary antibodies were from DAKO A/S (catalog No. F205) and Jackson ImmunoResearch Laboratories Inc (No. 115-095-146). The 5-HT\(_{3A}\) receptor antibody was a kind gift from Dr Bryan Roth (Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio). Anti-α\(_{1A}\)-adrenergic receptor (No. sc-1475, 1:50) and the secondary peroxidase-conjugated mouse anti-goat antibody (1:10 000) were obtained from Santa Cruz. AlF\(_4^-\) (30 µmol/L) was obtained by mixing 20 mmol/L NaF with 60 µmol/L AlCl\(_3\). Cirazoline hydrochloride and calyculin A were from ICN. Fura 2-AM and fluo 4-AM were from Molecular Probes. All other reagents were from Sigma Chemical Co.

Cholesterol Analysis
After equilibration, segmented arteries were incubated with or without 10 mmol/L mβcd for 1 hour at 37°C. Pelleted segments were frozen in liquid nitrogen. Freeze-dried pellets were weighed, and total cholesterol was measured fluorometrically.

Statistical Analysis
Summarized data are presented as mean±SEM. The Student t test or Wilcoxon matched pairs test was used for statistical comparisons. A value of P<0.05 was considered significant; n denotes the number of preparations. A minimum of 3 different animals was used in all sets of experiments.

Results
Endothelium-denuded rings of rat tail artery were contracted with high K\(^+\) (60 mmol/L), the α\(_{1A}\)-adrenergic–selective agonist cirazoline (0.3 µmol/L), 5-HT (1 µmol/L), or arginine vasopressin (AVP, 0.1 µmol/L). These responses, taken as control (Figure 1), were 90% to 100% of those to a maximally active concentration (E\(_{max}\)) of the respective agonist and were stationary for 3 hours (data not shown). After relaxation (25 minutes), preparations were treated for 1 hour with mβcd (10 mmol/L), which reduced their cholesterol contents by 21% (0.56±0.024 versus 0.71±0.026 [control] µg/mg dry tissue, n=12, P<0.001). Contraction was thereafter elicited again (Figure 1A and 1B). Contractions by endothelin-1 (10 nmol/L, giving ≅50% of E\(_{max}\) response), AlF\(_4^-\) (30 µmol/L, giving ≅90% of E\(_{max}\)), or the myosin phosphatase inhibitor calyculin A (3 µmol/L, giving ≅100% of E\(_{max}\)) were poorly reversible; thus, force after cholesterol extraction was compared with controls run in parallel. Summarized data from at least 8 individual experiments (Figure 1C) show that mβcd treatment selectively reduces 5-HT, AVP, and endothelin-1 responses.

Cumulative dose-response relations were determined in mβcd-treated and control preparations. For 5-HT, the dose-response relation was shifted ≅10-fold to the right after extraction of cholesterol, and force at saturating concentrations of 5-HT was reduced by 50% (please see online Figure IA, available at http://atvb.ahajournals.org). Neither of these effects was observed with the α\(_{1A}\)-agonist or with AlF\(_4^-\). Responsiveness to 5-HT was restored after cholesterol had been added back in complex with mβcd (please see online Figure IB). This recovery
5-HT are critically dependent on extracellular Ca\(^{2+}\) and L-type voltage-activated Ca\(^{2+}\) channels, inasmuch as verapamil (1 μmol/L) completely blocks 5-HT-induced force (not shown). Experiments in Ca\(^{2+}\)-free extracellular solution (0.5 mmol/L EGTA) were used to examine the effect of cholesterol depletion on Ca\(^{2+}\) release from intracellular stores. After depletion, the Ca\(^{2+}\)-release response with α₁-adrenergic stimulation (3 μmol/L cirazoline) increased (expressed as increase over baseline, from 48±8 to 129±3 mmol/L, n=4, P<0.05). Similarly, the increase in [Ca\(^{2+}\)]c, induced by caffeine (20 mmol/L) was significantly larger in depleted preparations (210±47 versus 47±2 mmol/L, n=4, P<0.05). These data suggest a higher Ca\(^{2+}\) loading of the sarcoplasmic reticulum (SR) in cholesterol-depleted preparations. Interestingly, Ca\(^{2+}\) release by 5-HT (10 μmol/L) was unchanged after cholesterol extraction (68±12 versus 60±9 mmol/L, n=9). To exclude the possibility that an increase in stored Ca\(^{2+}\) would mask an effect of mβcδ on α₁-adrenergic responses, tissues were treated with thapsigargin and caffeine to deplete Ca\(^{2+}\) from intracellular stores. This did not reveal any difference (control versus mβcδ treatment) in α₁-adrenergic responses in the presence of extracellular Ca\(^{2+}\) (n=8, data not shown).

Individual smooth muscle cells in the arterial wall display asynchronous Ca\(^{2+}\) oscillations on agonist stimulation, which allow for spatial and temporal coding of [Ca\(^{2+}\)]c, and most likely contribute to the control of force.\(^{20,21}\) Their frequency and amplitude, together with the fraction of oscillating cells and interwave [Ca\(^{2+}\)]c, form the basis for the global [Ca\(^{2+}\)]c signal measured over the vascular wall. Ca\(^{2+}\) wave activity was determined by use of confocal laser scanning microscopy of fluo 4–loaded mβcδ-treated and control arteries. The number of cells exhibiting waves during stimulation with either cirazoline (0.1 μmol/L) or 5-HT (0.3 μmol/L) was little affected by mβcδ treatment, whereas the mean wave frequency in treated vessels was 37% lower during 5-HT stimulation and marginally (14%) lower during activation with cirazoline (please see online Figure IIIB and IIC). The wave amplitude was decreased by 23% with 5-HT, whereas no effect was observed with cirazoline. This suggests that the capacity for Ca\(^{2+}\) wave generation is largely maintained after mβcδ treatment and that the decrease in activity during 5-HT stimulation is due to an upstream defect.

Light microscopic images of the tail artery appeared normal; this was also the case after force registration and cholesterol extraction (not shown). Immunofluorescence signals from 2 caveola-specific proteins, caveolin-1 and cave-p60,\(^{15}\) were found along the smooth muscle cell surface (please see online Figure IIIA and IICC), available at http://atvb.ahajournals.org). Distribution of caveolin-2 was similar, but labeling was weaker. Caveolin-3 was not detected. Cholesterol extraction had no detectable effect on the distribution of any of these proteins (please see online Figure IIIB and IIDD).

By electron microscopy, we ensured that no tissue damage was induced during force registration, apart from the luminal surface where the endothelium had been removed (not shown). In the nondepleted preparations, the smooth muscle plasma membrane contained numerous, often clustered, caveolae (Figure 2A). Cholesterol depletion (1 hour) caused an overall change in the appearance of caveolae. After depletion, the individual caveolae had either disappeared (had been flattened) or were...
shallow with wide openings (Figure 2B and 2C). Furthermore, the Golgi complex appeared disorganized but was the only other organelle affected. Traditional signs of tissue damage were not induced by the limited 1-hour cholesterol extraction. After cholesterol reloading of the depleted preparations, the number and morphology of caveolae appeared similar to that of muscle cells in control preparations (Figure 2D).

The levels of the 5-HT$_{2A}$ receptor and caveolin-1 were found to be unaffected by cholesterol extraction in Western blot experiments (Figure 3A and 3B). In silver-stained gels, the protein pattern of depleted and control preparations appeared to be similar (not shown). By subcellular fractionation on a sucrose density gradient, we found that the 5-HT$_{2A}$ receptor cofractionated with caveolin-1 and Cav-p60 in fractions 5 and 6 (Figure 3C). On the other hand, the $\alpha_{1A}$-adrenergic receptor was present in fractions 9 and 10 (Figure 3C).

**Discussion**

A large number of signaling molecules are copurified with caveolin in subcellular fractions prepared on sucrose gradients, suggesting that caveolae are involved in cellular signaling. Additional data, such as coimmunoprecipitation or double immunolabeling with caveolin, have firmly established the presence of several signaling proteins in caveolae. Those of relevance for vascular physiology include endothelial NO synthase, Ca$^{2+}$-ATPase, the endothelin receptor, and the platelet-derived growth factor receptor. Impaired endothelin-induced vascular contractility in caveolin-1 knockout mice provides further independent support for a role of caveolae in G-protein–coupled receptor signaling. In the present study, we demonstrate that cholesterol extraction impairs the function of 3 receptors, including that for endo-
localized in caveolae, but the ultrastructural localization of receptors, respectively. Although a rigorous morphometric quantification of the present findings with those from caveolin-1 deficient mice suggests a causal relationship between caveolae and arterial tone. Contents are given relative to the optical density/mm² of the control band (100%) on the same blot (n=8, P<0.05 for both comparisons). Identical amounts of tissue dry weight (60 μg) were loaded in each lane. C, Coenrichment of 5-HT₂A receptors, but not α₁A-adrenergic receptors, with caveolin-1 and cavin-6 in sucrose density fractionations of nontreated tail artery. Proteins in a homogenate of tail artery (4 rats per experiment) were separated on a discontinuous (5%, 35%, 45%) sucrose gradient, and equal volumes of each fraction (1 to 10) were loaded on the gel. The Western blots shown are representative of 3 independent fractionations.

Because caveolin-1 contents and localization appeared normal, these results imply that receptor functionality does not depend on caveolin-1, per se, but rather on a unique membrane organization governed by the interaction between cholesterol and other constituents of the membrane, such as caveolin-1. Although a rigorous morphometric quantification was not performed, the morphology of caveolae was clearly and reversibly altered by cholesterol extraction, with different degrees of flattening, indicating disruption of the caveolar structure. Although the present study did not distinguish the role of caveolae from that of other cholesterol-dependent structures in the plasma membrane, such as rafts, the combination of the present findings with those from caveolin-1-deficient mice suggests a causal relationship between caveolar structure and endothelin responses.

The contractile responses to endothelin-1, 5-HT, and vasopressin in the rat tail artery are mediated by ET₄, 5-HT₃A, and V₁ receptors, respectively. The ET₄ receptor was reported to be localized in caveolae, but the ultrastructural localization of 5-HT₃A or V₁ receptors has, to our knowledge, not been described in caveolin-1-expressing cells. However, the membrane localization and dynamics of internalization of the 5-HT₃A receptor was elegantly visualized in HEK-293 cells, which express little caveolin-1. Thus, caveolae are not required for membrane targeting of the receptor, whereas its function in intact arterial smooth muscle seems to require cholesterol, correlating with intact caveolar structure.

α₁A-Adrenergic receptors were found to be present primarily in fractions 9 and 10. This presumably explains the persistence of cirazoline-induced responses after cholesterol extraction and is consistent with findings of unaltered contraction in response to phenylephrine in rabbit aortas after cholesterol extraction and in aortas of caveolin-1-deficient mice. In cultured rat aortic cells, radioligand binding to α₁A-adrenergic receptors was found in caveolin-3–containing fractions. In our preparations, caveolin-3 was not detectable by immunofluorescence staining, and receptors were detected by using a subtype-specific α₁A-adrenergic antibody. This, as well as the marked phenotypic effects of culture on smooth muscle cells, potentially explains the discordant results, which suggest that the localization of receptors may be tissue and subtype specific.

The present results allow several conclusions regarding the steps in signaling affected by cholesterol depletion. Because adrenergic contractions are unaffected, it is unlikely that the contractile mechanism is involved. Furthermore, as also reported by Lohn et al., L-type channel activation by membrane depolarization seems to function normally in cholesterol-depleted preparations, because responses to depolarization by high K⁺ were unaffected. Thus, the impaired 5-HT–induced elevation of [Ca²⁺], after extraction of cholesterol seems to be due to a defect upstream from Ca²⁺ channel activation, presumably leading to reduced membrane depolarization after agonist stimulation. Moreover, because direct G-protein activation with the use of AlF₃ caused normal responses after cholesterol extraction, signaling downstream from G-protein activation was not affected.

Agonist-stimulated Ca²⁺ wave activity, which depends on recurring inositol trisphosphate (InsP₃)-mediated Ca²⁺ release from the SR, was selectively affected by cholesterol extraction, with a larger decrease for 5-HT than for cirazoline stimulation, consistent with fura 2 measurements of global [Ca²⁺]. This suggests that InsP₃ formation by 5-HT stimulation may be compromised by cholesterol depletion, as may also be inferred from a comparison of agonist-induced Ca²⁺ release responses in Ca²⁺–free medium. The mβcd treatment causes increased basal [Ca²⁺], and, probably as a consequence, increased Ca²⁺ loading of the SR. This is evidenced by the increased response to caffeine, an agent that acts directly at ryanodine receptors on the SR. Because responses to cirazoline were also increased after cholesterol depletion but those to 5-HT were not, it may be inferred that InsP₃ production by the latter agonist had been reduced.

Treatment with mβcd might have caused a slight increase of membrane permeability, which causes increased basal [Ca²⁺], and contractile tone, but otherwise, it does not seem to impair tissue function. Because Ca²⁺ efflux during repolarization was unaffected by cholesterol depletion, Ca²⁺ pumps were not inhibited. The basal tone, although sensitive to extracellular Ca²⁺, was resistant to a variety of Ca²⁺ channel blockers and, thus, presumably not associated with decreased activity of Ca²⁺ sparks and spontaneous transient outward currents, as reported for mβcd-treated cells and after caveolin-1 knockout.

Our results suggest that the signaling step impaired by cholesterol depletion is located either to ligand-receptor or to receptor–second-messenger interaction. Both steps could poten-
tially be influenced by local variations in membrane fluidity imposed by caveolin-cholesterol interactions. Because the amount of receptor protein was not affected by cholesterol extraction, the localization of the receptor in cholesterol-rich regions, such as caveolae, seems to be necessary for proper function.

Acknowledgments

The study was supported by the Swedish Research Council (71X-28), by Magnus Bergvalls Stiftelse, and by postdoctoral fellowships from Svenska Sällskapet fór Medicinsk Forskning (to K.S.) and the faculty of Umeå University Medical School. The study was also supported by Magnus Bergvalls Stiftelse, and by postdoctoral fellowships from Svenska Sällskapet fór Medicinsk Forskning (to K.S.) and the Danish Medical Research Council (to M.V.). We thank M. Danielsen and L. Immerdal for cholesterol measurements, H. Hadberg, M. Soberg, and P.S. Thomsen for microscopic preparations, and B. Risto for skilful photographic work.

References

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Arterioscler Thromb Vasc Biol. 2002;22:1267-1272; originally published online May 23, 2002; doi: 10.1161/01.ATV.0000023438.32585.A1
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Figure I. Effect of methyl-β-cyclodextrin on dose-response relations and reversal of serotonergic contractility by cholesterol. A, dose-response relations for 5-HT, the α1-agonist cirazoline, and AlF₄⁻ in control (filled circles) and mβcd treated preparations (open circles). Dose-response relations to AlF₄⁻ were obtained by varying [NaF] at 30 µM AlCl₃. B, reversal of serotonergic contractility by cholesterol added in complex with mβcd (10 mM). C shows summarized data from the experiments in panel B. The first contraction after extraction represents 0 min. Filled circles: preparations treated with mβcd and subsequently transferred to HEPES buffered Krebs. Open circles: preparations treated with mβcd and subsequently reloaded with cholesterol (n=8 for both).
Figure II. Global and local intracellular Ca\(^{2+}\) elevation in response to 5-HT, membrane depolarization and \(\alpha_1\) stimulation after cholesterol depletion. In A, Fura 2 loaded control (black line) and depleted (dotted line) preparations were stimulated by high-K\(^+\) and 5-HT (1 \(\mu\)M). In B, confocal imaging of InsP\(_3\)-dependent Ca\(^{2+}\) waves in four cells in the media of a Fluo 4 loaded artery stimulated with 0.1 \(\mu\)M cirazoline. C shows summarized data for mean fraction of cells exhibiting wave activity (left), wave frequency (middle) and wave amplitude (right) during stimulation with cirazoline and 5-HT in control (black bars) and depleted (white bars) arteries. ***P<0.001, *P<0.05, n>50 cells for all.
Figure III. Cholesterol depletion has no apparent effect on the distribution of caveolar proteins at the level of light microscopy. Paraffin sections of rat tail artery prepared for indirect immuno-fluorescence microscopy before (A, C) and after cholesterol depletion (B, D). A and B are labeled for caveolin-1. C and D are labeled for cav-p60. When the muscle cells are not cut at right angles the labeling appears smeared.