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 Ca\(^{2+}\) handling were evaluated. In cholesterol-depleted preparations, the force responses to \(\alpha_1\)-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 \(\mu\)mol/L AlCl\(_3\) were unaffected. In contrast, responses to 5-hydroxytryptamine (5-HT), vasopressin, and endothelin were reduced by >50%. The rise in global intracellular free Ca\(^{2+}\) concentration in response to 5-HT was attenuated, as was the generation of Ca\(^{2+}\) 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-HT\(_{2A}\) receptor and of caveolin-1 were unaffected by cholesterol extraction. Sucrose gradient centrifugation showed enrichment of 5-HT\(_{2A}\) receptors, but not \(\alpha_1\)-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 B\(_2\) receptors). Cholesterol-rich membrane domains may be considered as possible physical platforms for the coding of intracellular signals. To investigate this, intracellular Ca\(^{2+}\) 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 \(\alpha_1\)-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 m\(_{-}\)-cyclic adenosine monophosphate (cAMP) 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 4% formaldehyde and 0.1% 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 then incubated with fluo 4-AM for 90 minutes after exposure to 5-HT. Control sections omitting the primary antibody were devoid of specific fluorescence signal. 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.

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 (686, 1 μg/mL), 5-hydroxytryptamine (5-HT)\(_{2A}\) receptor (1:500), or Cav-p60 (1 μg/mL). Peroxidase-conjugated secondary antibodies were used, and peroxidase activity was detected by chemiluminescence (Pierce).

Preparation of Caveolin-Enriched Fractions

Three to 5 freshly dissected tail arteries were transferred to 1 mL of 0.1 mol/L NaCl, 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).

Cholesterol Analysis

After equilibration, segmented arteries were incubated with or without 10 mmol/L mβcld 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\(^{19}\) (0.3 μmol/L), 5-HT (1 μmol/L), or arginine vasopressin (AVP, 0.1 mmol/L). These responses, taken as control (Figure 1), were 90% to 100% of those to a maximally active concentration (E\(_{\text{max}}\)) of the respective agonist and were stationary for 3 hours (data not shown). After relaxation (25 minutes), preparations were treated for 1 hour with mβcld (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 then elicited again (Figure 1A and 1B). Contractions by endothelin-1 (10 nmol/L, giving ≈50% of E\(_{\text{max}}\) response, AlF\(_{4}^-\) (30 μmol/L, giving ≈90% of E\(_{\text{max}}\)), or the myosin phosphatase inhibitor calyculin A (3 μmol/L, giving ≈100% of E\(_{\text{max}}\)) 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 mβcld treatment selectively reduces 5-HT, AVP, and endothelin-1 responses. Cumulative dose-response relations were determined in mβcld-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 mβcld (please see online Figure IB). This recovery
whereas the response to 1 mM H11005 nmol/L, n (175 mmol/L, n = 14, P < 0.001). The increase in [Ca2+]i induced by caffeine (20 mmol/L) was significantly larger in depleted preparations (210 to 47 ± 2 nmol/L, n = 4, P < 0.05). These data suggest a higher Ca2+ loading of the sarcoplasmic reticulum (SR) in cholesterol-depleted preparations. Interestingly, Ca2+ release by 5-HT (10 mmol/L) was unchanged after cholesterol extraction (68 ± 12 versus 60 ± 9 mmol/L, n = 9). To exclude the possibility that an increase in stored Ca2+ would mask an effect of mβcδ on α1-adrenergic responses, tissues were treated with thapsigargin and caffeine to deplete Ca2+ from intracellular stores. This did not reveal any difference (control versus mβcδ treatment) in α1-adrenergic responses in the presence of extracellular Ca2+ (n = 8, data not shown).

Individual smooth muscle cells in the arterial wall display asynchronous Ca2+ oscillations on agonist stimulation, which allow for spatial and temporal coding of [Ca2+]i, and most likely contribute to the control of force.20,21 Their frequency and amplitude, along with the fraction of oscillating cells and interwave [Ca2+]i, form the basis for the global [Ca2+]i signal measured over the vascular wall. Ca2+ wave activity was determined by use of confocal laser scanning microscopy of fluor 4-loaded mβcδ-treated and control arteries. The number of cells exhibiting waves during stimulation with either cirazoline (0.1 mmol/L) or 5-HT (0.3 mmol/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 IIB 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 Ca2+ 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 cav-p60,15 were found along the smooth muscle cell surface (please see online Figure IIIA and IIC). 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 IIB and IIC).

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

Figure 1. Cholesterol depletion selectively reduces agonist responses in the rat tail artery. A and B. Original recordings of force in deendothelialized segments of the rat tail artery contracted with 60 mmol/L K+ (A) or 1 μmol/L 5-HT (B). After stable contractions had been attained, cholesterol was extracted by treatment with 10 mmol/L mβcδ for 1 hour at 37°C. Thereafter, the same agonist was again used to elicit contraction. C. Summarized data on force elicited by the phosphatase inhibitor calyculin A (3 μmol/L) or 1 μmol/L cirazoline (0.3 μmol/L), 5-HT (1 μmol/L), AVP (0.1 μmol/L), endothelin-1 (10 mmol/L), and AF698 (30 μmol/L) before (solid bars) and after (open bars) cholesterol depletion. ***P < 0.001 by Student t test (n = 8).

was not spontaneous, inasmuch as extracted preparations run in parallel but not receiving cholesterol continued to weaken (please see online Figure IC). After recovery, force was maintained for at least 1 further contraction without continuing incubation with cholesterol.

[Ca2+]i was determined by the fura 2 technique (please see online Figure IIA, available at http://atvb.ahajournals.org). Basal [Ca2+]i, was significantly increased in cholesterol-depleted preparations (121 ± 18 [μmol/L] versus 37 ± 5 [control] n = 14, P < 0.001). This was paralleled by an increased Ca2+-dependent basal tone (7.1 ± 1.1% [μmol/L] versus 0.5 ± 0.5% [control] at 1 hour, n = 34), which was insensitive to inhibitors of L-type Ca2+ channels (1 or 10 μmol/L verapamil), store-operated Ca2+ channels (10 μmol/L SKF 96365 and 1 mmol/L Ni2+), and nonselective cation channels (100 μmol/L LOE 908). Furthermore, Ca2+ extrusion mechanisms were apparently not affected by the treatment, because the rate of decline in [Ca2+]i, on repolarization from high K+ in Ca2+-free solution was equal in mβcδ-treated and control arteries (half-time 28.5 ± 1.2 versus 23.5 ± 2.6 seconds, n = 10).

Average [Ca2+]i during depolarization with high K+ (328 ± 21 [μmol/L] versus 267 ± 19 [control] n = 12, P = NS) or 0.3 μmol/L cirazoline (212 ± 25 [μmol/L] versus 219 ± 30 [control] n = 4, P = NS) was not affected by cholesterol depletion, whereas the response to 1 μmol/L 5-HT was drastically reduced (175 ± 17 [μmol/L] versus 333 ± 40 [control] n = 10, P < 0.01; please see online Figure IIA). Contractile responses to 5-HT are critically dependent on extracellular Ca2+ and L-type voltage-activated Ca2+ channels, inasmuch as verapamil (1 μmol/L) completely blocks 5-HT–induced force (not shown). Experiments in Ca2+-free extracellular solution (0.5 mmol/L EGTA) were used to examine the effect of cholesterol depletion on Ca2+ release from intracellular stores. After depletion, the Ca2+-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 [Ca2+]i, 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 Ca2+ loading of the sarcoplasmic reticulum (SR) in cholesterol-depleted preparations. Interestingly, Ca2+ release by 5-HT (10 mmol/L) was unchanged after cholesterol extraction (68 ± 12 versus 60 ± 9 mmol/L, n = 9). To exclude the possibility that an increase in stored Ca2+ would mask an effect of mβcδ on α1-adrenergic responses, tissues were treated with thapsigargin and caffeine to deplete Ca2+ from intracellular stores. This did not reveal any difference (control versus mβcδ treatment) in α1-adrenergic responses in the presence of extracellular Ca2+ (n = 8, data not shown).

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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\textsubscript{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\textsubscript{2A} receptor cofractionated with caveolin-1 and Cav-p60 in fractions 5 and 6 (Figure 3C). On the other hand, the \(\alpha\textsubscript{1A}\) -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.\(^7,8,22\) 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\textsuperscript{2+}-ATPase, the endothelin receptor, and the platelet-derived growth factor receptor.\(^22\) Impaired endothelin-induced vascular contractility in caveolin-1 knockout mice\(^23\) 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-

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**Figure 2.** Micrographs (by electron microscopy [EM]) of the medial smooth muscle cells (SMCs) in tail arteries. Plasma membrane areas of SMCs from different preparations are shown. A, Normal \(\Omega\)-shaped caveolae (arrows) are shown in an SMC from a control preparation. Arrowheads indicate Golgi apparatus; M, mitochondrion; N, nucleus; and F, filaments. B and C, Cholesterol-depleted SMCs are shown. Caveolae are less numerous and, when present, are opened to a variable degree. The Golgi apparatus is disorganized. D, Normal caveolae are seen after reloading with cholesterol.
localized in caveolae, but the ultrastructural localization of the 5-HT2A 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, membrane organization governed by the interaction between cholesterol and other constituents of the membrane, such as caveolin-1. Although a rigorous morphometric quantification of caveolin-1 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+]i 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 AIF2 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+]i. This suggests that InsP3 formation by 5-HT stimulation may have been 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+]i, 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+]i, 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.

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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 α1 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 µM). 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 µM 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.
control  mβcd treated

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.