Functional Compartmentation of Endothelial P2Y Receptor Signaling

Robert A. Kaiser, Brian C. Oxhorn, Gracie Andrews, Iain L.O. Buxton

Abstract—The presence of multiple receptors for disparate nucleotides on endothelial cells makes it unclear how the endothelium differentiates among these signals. We propose that endothelial P2Y receptors are organized into cholesterol-rich signaling domains, such as caveolae and respond to nucleotide agonists by mobilizing intracellular calcium. Treatment of endothelial cells with 5 mmol/L β-methyl-cyclodextrin prevents calcium release in response to the nucleotide receptor agonists 2-methylthio-ATP, ATP, ADP, and UTP, but not the kinin receptor agonist bradykinin, suggesting that depletion of membrane cholesterol disrupts signaling at P2Y receptors and that bradykinin receptors are not prelocalized to cholesterol microdomains in these cells. Direct measurement of cholesterol content after β-methyl-cyclodextrin treatment of aortic rings reveals a concentration-dependent depletion of cholesterol that parallels functional antagonism of P2Y-mediated relaxation. Nucleotide- and bradykinin-mediated relaxation is disrupted by 5 to 15 mmol/L β-methyl-cyclodextrin treatment or 1 to 10 μg/mL filipin III in a concentration-dependent fashion. Norepinephrine contracted aorta treated with A23187 relaxes in an endothelium-dependent fashion despite depletion of 84% of membrane-extractable cholesterol. These data indicate that in the basal state, P2Y receptors but not the kinin receptor may be compartmented to cholesterol-dependent signaling domains in guinea pig endothelium and that cholesterol-rich microdomains in these cells can respond to intracellular calcium in an agonist-specific manner. We suggest that the functional organization of cholesterol-rich signaling microdomains allows agonist-specific responses to increases in intracellular calcium and that this property may be a general phenomenon that permits cells to respond disparately to agonists that may signal through common calcium release pathways. (Circ Res. 2002;91:xxx-xxx.)

Key Words: nucleotides, 2-methylthio-ATP, caveolae, calcium, filipin

Vascular endothelial cells express receptors that recognize extracellular nucleotides1 in a fashion consistent with a role for such receptor signaling in the maintenance of blood flow.2 Receptors for nucleotides exist in 2 major families: P2Y (GTP-binding protein coupled 7-transmembrane domain receptors) and P2X (receptors ion channels).3 There are at least 6 cloned P2Y receptors, and 7 cloned P2X receptors that are categorized by their ligand selectivity.4,5 Most P2Y receptors are capable of mediating responses to several nucleotides, resulting in multiple receptors enjoying overlapping ligand preferences.3 For example, guinea pig aortic endothelial cells (GPAECs) possess both P2Y1 and P2Y2 receptors activated by ATP and ADP as well as P2Y1 and P2Y2 receptors that respond to UTP.6,6 Furthermore, the same GTP-binding protein, Gq/11,6 is responsible for signaling the release of calcium from intracellular stores in response to stimulation by each of these receptors on the same cell5,7 with subsequent activation of nitric oxide synthase (eNOS), leading to cGMP-dependent relaxation of the underlying vascular smooth muscle.8 Thus, it is wholly unclear if, or if so how the cell regulates its response to the simultaneous presence of multiple nucleotides such as occurs when platelets degranulate.

When first presented by us in 1983,9 subcellular compartmentation of receptor/G-protein–coupled signaling was not understood. Although the discovery of numerous details of signaling regulation such as kinase anchoring proteins and the role of the cytoskeleton have provided a context for studies of signaling compartments, the discovery of cholesterol-rich regions in the plasma membrane10 and their recognition as signaling domains offers a significant breakthrough in our understanding of how cells organize their response to numerous extracellular stimuli.11,12 Many signaling pathways are now known to be compartmented by caveolae, and to date, associated signaling molecules include bradykinin (BK) receptors,13,14 muscarinic acetylcholine (ACh) receptors,15 receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor,16 and G proteins such as Gq, and Ga.17 Of particular relevance to endothelial function, the link between eNOS and the caveolar protein caveolin-1 has implications for vascular regulation. Because endothelial P2Y receptors use NO as their primary effector mediating relaxation,18 and

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because the caveolin-1/eNOS complex is a key inhibitory regulator of NO production in the endothelium,19 we sought to investigate the hypothesis that P2Y receptors are colocalized in cholesterol-rich membrane microdomains.

Compartmentation of the endothelial response to nucleotides would further our understanding of the Nucleotide Axis Hypothesis, which proposes a framework by which nucleotides act as blood-borne autocrine and paracrine hormones capable of mediating vessel tone on a moment-to-moment basis.2 Our work and that of others has demonstrated that endothelial cells respond to hormonal stimuli and shear stress21 by releasing UTP22 and ATP into the vessel lumen. ATP can then act on endothelium at, and downstream from, the site of its release to signal vasodilation at P2Y receptors as ATP per se and again after its metabolism to ADP on the surface of endothelium.2,23 The subsequent regeneration of ATP from ADP on the surface of endothelium by the ecto-nucleoside diphosphate kinase (eNDPK)2,24 propagates vasodilatation within the blood vessel by maintaining ATP, and thus ADP, concentrations locally. The eventual metabolism of released ATP to adenosine (ADO) by the time flow reaches resistance vessels further subserves dilation,2 yielding increased blood flow regionally. The precise role of UTP in the Axis is less well known but intriguing based on its role as a phospholipid donor in the eNDPK reaction converting ADP to ATP, and its activity at P2Y4.

In this study, we present evidence in guinea pig aorta that nucleotide-mediated endothelial cell calcium mobilization and the resulting relaxation of vascular smooth muscle are sensitive to both the removal of membrane cholesterol by β-methyl-cyclodextrin (β-mCD) and the sequestration of cholesterol by filipin. Our results show for the first time that P2Y receptors are functionally compartmented to cholesterol-rich microdomains in GPAECs and that such microdomains act in an agonist-specific manner to increase the sensitivity of intracellular calcium. We suggest a model whereby endothelium may differentiate nucleotide and other stimuli depending on organization and regulation of the microdomain complement of receptors and their coupling. Furthermore, in an effort to assess the results of β-mCD treatment of small blood vessel segments (3 to 4 mg wet wt), we detail a rapid and sensitive method for the determination of tissue cholesterol using HPLC.

Materials and Methods

Contractile Experiments

Descending thoracic aortae, removed from female guinea pigs after their euthanasia under an approved protocol, were dissected into 4- to 6-mm rings and suspended on triangles in organ baths (Radnoti) between a fixed point and a force transducer (Kent Scientific) for the measurement of tension as described.6 Tissues were equilibrated in Krebs bicarbonate buffer (pH = 7.4) for 1 hour and precontracted twice with 60 mmol/L potassium. Drugs were diluted 1:1000 and only tissues that contracted to 300 mmol/L norepinephrine (NE) and subsequently relaxed to 100 mmol/L 2-methylthio-ATP (2-MeS-ATP) were used. Endothelial cell membrane cholesterol was depleted by treating whole tissues with β-mCD (5 to 15 mmol/L) for 1 hour at 37°C.25 The treatment was washed out and the tissue recovered for 10 minutes. In order to establish that the results observed with β-mCD were due to specific effects on membrane cholesterol, parallel studies were performed with filipin (1 to 10 mg/L; 30 minutes), which is known to sequester cholesterol within the membrane.25

Endothelial Cell Isolation and Calcium Imaging

GPAECs were prepared by primary dispersion from dissected aortae7 and grown in culture for 1 week. The maintenance of endothelial phenotype in culture was independently confirmed by flow cytometric analysis of CD31 expression and binding of UEA.2 GPAECs were grown to 25% confluence, washed with a MOPS buffer and loaded with Fura-2 AM (2 μmol/L; Molecular Probes).17 Agonists were added by removing 0.5 vol of buffer and replacing it with agonists at 2X in 0.5 vol buffer. This procedure was found to be preferable to either direct concentrated addition or continuous perfusion of agonists because it resulted in a more rapid response.2 For cholesterol depletion studies, cells were incubated with 5 mmol/L β-mCD for 1 hour. Control experiments revealed that cultured endothelial cells lose 54±1.1% of their total cholesterol content in the first 20 minutes under these conditions (detection limit 1.5×104 cells). In some experiments, addition of ionomycin followed by Mn2+ served to reference calcium transient size. Precise calibration of intracellular calcium concentrations was neither desirable nor performed.

Cholesterol Determination

Dissociated aortic rings (∼4 mg wet wt) were treated with 0, 5, 10, or 15 mmol/L β-mCD (1 hour 37°C) or 1, 5, 10 mg/L filipin III (30 minutes; 37°C) and extracted with 100 μL of CHCl3 for 2 hours. Tissues were vortexed every 20 minutes to ensure uniform CHCl3 exposure. Residue was resuspended and the extract dried at 80°C. Residue was resuspended in 100 μL of methanol:acetonitrile 30:70 (v/v) and analyzed by HPLC.2 Briefly, 10 to 20 μL of resuspended, filtered vessel extract was injected onto a C18 column (46 x 100 mm; 5 μm; 35°C) and eluted over 10 minutes with methanol:acetonitrile 30:70 (v/v). The elution profile (A205) was recorded and standard retention (5.5±0.05 minutes) used to construct a standard curve (area under the curve [AUC]) from which unknown cholesterol contents were determined. Spiking of duplicate vessel samples was used to confirm our ability to detect cholesterol in real samples with a limit sensitivity of 300 pg. Amounts of cholesterol were normalized to wet tissue weight.

Tissue Histology

Guinea pig aortae were fixed in 4% paraformaldehyde for 1 hour. Vital dye-stained samples were first incubated at 37°C for 45 minutes in 25 μmol/L Cell Tracker Green (Molecular Probes) and then fixed. Tissues were infiltrated with a suroce series in PBS of 5% 1 hour, 15% 4 hours, 15% and 7.5% gelatin overnight 37°C. Tissues were embedded in gelatin for cryosectioning at 10 μm on a Leica CM3050 microtome and arranged on glass slides. For hematoxylin/eosin staining, gelatin was melted with PBS (50°C). Slides were fixed in 3% acetic acid in 95% MeOH, tap water rinsed, and stained with hematoxylin and eosin. Vital dye-stained tissues were counterstained with DAPI (4′,6-diamidino-2-phenylindole). Slides were dehydrated through an EtOH series and mounted with xylene in Eukitt media (Electron Microscopy Sciences). Both bright-field and fluorescent images were obtained with a Leica DMR microscope and LEI-750 digital camera with Optronics software.

Electron Microscopy

Vessel segments from physiological experiments treated with β-mCD or control as described above were fixed overnight at 4°C with a solution containing 2.5% glutaraldehyde (v/v), 2.0% paraformaldehyde (wt/vol), and 1.5% sucrose (wt/vol) dissolved in cacodylate buffer (0.1 mol/L, pH 7.3). Samples were washed with cacodylate buffer (0.1 mol/L) containing sucrose (1.5% wt/vol for 1 hour) and postfixed with osmium tetroxide (1% wt/vol) for 1 hour. Samples were dehydrated through a graded series of alcohols and embedded in Spurr’s low viscosity resin. Ultrathin sections were double stained with alcoholic uranyl acetate and lead citrate and...
Failure of GPAECs to respond to nucleotide agonists is not equilibration for as long as 90 minutes (data not shown). Nucleotide-Mediated Calcium Signaling Is Sensitive to β-mCD

Aortic rings contracted with 1 μmol/L NE relaxed to addition of the P2Y₁ receptor agonist 2-MeS-ATP (100 nmol/L; Figure 2A). After incubation of the vessel in 15 mmol/L β-mCD for 1 hour, responsiveness to 2-MeS-ATP is severely compromised (Figure 2B), whereas the control tissue relaxes to P2Y stimulation. Similar results are observed for ATP, ADP (Figure 3A), and UTP (Figure 3B) at 10, 1, and 100 μmol/L, respectively. In all tissues, tension returns to baseline on washout. Isoproterenol (ISO; 10 μmol/L) induced relaxation is insensitive to β-mCD pretreatment and is similar in both the control and treated tissues (Figure 3B). Total relaxation seen with ISO is additive with the relaxation observed in response to nucleotide agonists like UTP. Contractions observed to 60 mmol/L potassium are not affected by treatment with β-mCD (not shown).

Effects of β-mCD and Filipin Are Concentration-Dependent

With the observation that β-mCD can reduce the agonist-mediated relaxation observed in guinea pig aorta, we sought to determine if there is a concentration-dependence to the effect of both β-mCD and filipin to uncouple nucleotide-mediated relaxation. Increasing concentrations of both β-mCD (5 to 15 mmol/L) and Filipin III (1 to 10 mg/L) reduced aortic relaxations to 100 μmol/L 2-MeS-ATP in NE-stimulated tissues (Figure 4A). The effect of β-mCD treatment was 5.1 ± 2.0%, 20.8 ± 3.3%, and 62.1 ± 2.7% reductions of relaxation at 5, 10, and 15 mmol/L, respectively, whereas filipin reduced observed relaxations by 17.6 ± 9.9%, 59.4 ± 9.9%, and 100% at 1, 5, and 10 mg/L, respectively (Figure 4B). Using HPLC analysis of matched vessel extracts, we determined that the concentration-dependent effect of β-mCD to reduce the endothelial-dependent relaxation to nucleotides is paralleled by decreased cholesterol content in

Results

Nucleotide-Mediated Calcium Signaling Is Sensitive to β-mCD

Endothelial cells exposed to nucleotides mobilize intracellular calcium as mediated by P2Y receptor activation and G protein–dependent signaling, and such responses are well known to desensitize rapidly. Addition of 2-MeS-ATP and ATP (10 μmol/L) elicit robust calcium responses mediated by P2Y₁ and P2Y₂ receptors, respectively (Figure 1A). Subsequent addition of 10 μmol/L ADP is ineffective due to desensitization. Addition of BK (3 nmol/L) causes calcium release despite nucleotide receptor desensitization (Figure 1A). After incubation with 15 mmol/L β-mCD for 1 hour to remove plasmalemmal cholesterol, addition of nucleotides fails to promote calcium release (Figure 1B), despite re-equilibration for as long as 90 minutes (data not shown). Failure of GPAECs to respond to nucleotide agonists is not the result of disruption of calcium signaling per se considering addition of 3 mmol/L BK to β-mCD–treated cells elicits a calcium response that is unchanged when compared with controls (Figure 1B).
Figure 2. In functional studies of aortic contraction/relaxation, (A) vessel rings contract to 1 \( \mu \text{mol/L} \) NE and relax in response to 2-MeS-ATP (100 nmol/L). B, One ring (CD) was stripped of cholesterol (see Materials and Methods) and the relaxation of both to 2-MeS-ATP was repeated. CD treatment attenuates P2Y receptor–mediated relaxation. Results are representative of separate observations in multiple vessel rings from 2 to 5 animals.

Figure 3. Aortic rings depleted of cholesterol (CD) and control tissues were contracted in the presence of 3 \( \mu \text{mol/L} \) NE. A, Control tissues relaxed in response to P2Y stimulation by ADP (1 \( \mu \text{mol/L} \)) and ATP (10 \( \mu \text{mol/L} \)), whereas CD tissues did not. B, Control but not CD-treated tissues relaxed to 100 \( \mu \text{mol/L} \) UTP acting at the P2Y \(_4\) receptor, although both tissues relaxed to \( \beta \)-adrenergic stimulation by isoproterenol (ISO, 10 \( \mu \text{mol/L} \)). Results are representative of separate observations in multiple vessel rings from 2 to 5 animals.
the tissues (up to 83.7±6.4%; Figure 4C) after 15 mmol/L β-mCD treatment. The effects of filipin are not paralleled by reductions of extractable cholesterol content, consistent with cholesterol sequestration rather than depletion.

Direct Visualization of the Effects of β-mCD on Aorta

Treatment with β-mCD has profound effects on nucleotide-mediated signaling in endothelium. To verify that the endothelium is indeed intact after the β-mCD treatment, aortic rings from tissue studies were examined (Figure 5). An intact endothelial layer is observed after hematoxylin-eosin staining in both control (Figure 5A) and β-mCD–treated tissue (Figure 5B). To determine the viability of the endothelial cell layer after β-mCD treatment, we double labeled aortic rings with DAPI and the vital dye Cell Tracker Green. In these preparations, the GPAECs are capable of sequestering the vital dye in both control (Figure 5C) and β-mCD–treated (Figure 5D) samples. Treated cells (Figures 5B and 5D), although showing some obvious endothelial disruption, do remain viable despite the absence of relaxation to nucleotides.

That β-mCD depletion of cholesterol selectively depletes signaling microdomains in endothelium is demonstrated by the loss of caveolar structures in endothelial membranes examined under electron microscopy. Micrographs of endothelial membrane in control vessels (Figure 5E) clearly show the presence of caveolae that are rare or absent in samples treated with 15 mmol/L β-mCD (Figure 5F).

ACh and BK Cause Contraction in Cholesterol-Depleted Aorta

Considering the ability of BK to elicit a calcium response in GPAECs regardless of treatment with β-mCD, we wanted to investigate the functional response to BK in intact vessels. NE-contracted control tissues respond to 1 μmol/L BK with a pronounced relaxation (Figure 6A) similar to that observed in response to P2Y agonists. Aortic rings pretreated with β-mCD (15 mmol/L) for 1 hour and contracted with NE respond to the same concentration of BK that relaxes control tissues by contracting consistent with a dysfunctional endothelium. The same result is observed in response to 10

Figure 4. Ability of β-mCD and filipin to attenuate P2Y receptor relaxation in NE-contracted aortic rings was determined to be concentration-dependent, whereas only β-mCD effects were paralleled by reductions in membrane cholesterol. Examination of the effect of β-mCD (CD5=5 mmol/L, CD 10=10 mmol/L, CD 15=15 mmol/L) and filipin (Fil1=1 mg/L, Fil 5=5 mg/L, Fil 10=10 mg/L) on nucleotide-mediated relaxation (A and B) and cholesterol content (C). Reduced relaxation was correlated with reduced cholesterol content in β-mCD–treated rings, but not filipin-treated rings. Significance (compared with control) is indicated as *P<0.05, **P<0.01, and ***P<0.001. Data are representative of replicate observations in 3 independent experiments.
Similar observations are obtained with 10 mg/L filipin (data not shown).

**Effects of β-mCD Are Mimicked by MRS-2179 and L-NAME**

To further investigate the mechanism of cholesterol depletion on endothelium-dependent relaxation and determine the viability of the endothelial cell layer in our tissues, we compared the responses of tissues in 4 treatment groups: cholesterol depleted (CD), P2Y1 antagonized (MRS2179), eNOS inhibited (L-NAME), and control aortic rings (Figure 7A). P2Y1 receptor–mediated relaxation was specifically antagonized by 3 μmol/L MRS2179 before (5 minutes) NE contraction.6,7 Endothelial NOS was inhibited by treatment of the aorta with 100 μmol/L L-NAME 10 minutes before NE addition. On achieving a stable response to NE, the P2Y1 agonist 2-MeS-ATP was added (100 nmol/L) to each treatment group (Figure 7B). Only the control sample was capable of achieving complete relaxation, whereas the β-mCD–treated sample displayed a small transient relaxation to 2-MeS-ATP. To determine the functional viability of the endothelium, we added the calcium ionophore A23187 (1 μmol/L; Figure 7C).

**Figure 5.** Histological sections were prepared from aortic rings (control: A, C, and E; β-mCD treated: B, D, and F) used in tissue bath experiments. Cross sections (10 μm) of aorta (lumen up) stained with hematoxylin-eosin (A and B) or DAPI/vital dye (C and D) were viewed through a bright-field objective at 50× magnification. Electron micrographs of the endothelial layer obtained at 18 000× (E and F) with insets (3×) are shown to demonstrate the presence (E) or absence (F) of caveolae. Images are representative of numerous observations from multiple tissues.

**Figure 6.** Aortic rings pretreated with β-mCD (CD) contract to common vasodilatory signals, indicating loss of endothelial function. A, Control NE-contracted tissue dilates to the addition of 1 μmol/L bradykinin, whereas β-mCD–pretreated tissues contract to the same stimulus. B, Similar observations are made to ACh additions (10 μmol/L), where control tissue responds by relaxing and β-mCD–treated rings contract. Traces are representative of replicate observations in 3 to 5 separate experiments.
Control tissue responded with enhanced relaxation that was only partially additive with the 2-MeS-ATP–induced relaxation, suggesting the NO dependence of both signaling mechanisms. Both the β-mCD– and MRS2179-treated aortae also relaxed to A23187, indicating that the endothelium is viable and that both perturbations disrupt 2-MeS-ATP signaling upstream of intracellular calcium release. L-NAME treatment blocks the endothelial response to A23187 but not the smooth muscle response (Figure 7C).

**Discussion**

Lipid-enriched signaling domains like caveolae and detergent-insoluble glycolipid-rich domains known as DIGs$^{27}$ are recognized to play roles in many cellular processes, especially receptor signal-transduction (for review, see Anderson$^{11}$). We have demonstrated for the first time that nucleotide P2Y receptor signaling is sensitive to perturbation of membrane cholesterol in GPAECs, implicating P2Y interaction with cholesterol-rich signaling domains like caveolae. The effect of cholesterol depletion on the action of P2Y receptors is independent of downstream signaling events such as release of intracellular calcium because it is specific for P2Y receptor signaling versus that of bradykinin. BK receptors translocate to cholesterol-rich signaling domains on addition of agonist$^{14,16}$ and before membrane cholesterol depletion, both BK and P2Y receptors signal calcium release. Because BK receptors (but not P2Y receptors) couple to calcium release after β-mCD treatment, we suggest that P2Y receptors are precoupled to cholesterol-rich domains, whereas BK receptors must see agonists to translocate but still can couple to calcium release without entering such domains. Indeed, this distinction may be supported by the fact that nucleotide responses desensitize rapidly although BK responses do not, as if prelocalization facilitates receptor desensitization. Furthermore, endothelium-dependent vasodilation is restored in β-mCD and filipin-treated tissues by addition of the calcium ionophore A23187$^{28}$ supporting the conclusion that such treatment renders the endothelium dysfunctional rather than nonfunctional. Nucleotide signaling by endothelium is one way that endothelial cells modulate blood vessel tone$^{7}$ on a moment-to-moment basis.$^{2,20,24}$ Our demonstration that disruption of endothelial signaling domains

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**Figure 7.** β-mCD treatment disrupts endothelial vasodilation upstream of intracellular calcium release. A, NE-contracted aortic rings relax to 100 nmol/L 2-MeS-ATP addition (control) in a manner sensitive to P2Y$_1$ antagonism (3 μmol/L MRS2179), eNOS inhibition (100 μmol/L L-NAME), and cholesterol depletion (15 mmol/L β-mCD). Tissues with antagonized P2Y$_1$ or disrupted cholesterol still relax to the addition of 1 μmol/L A23187, indicating functional endothelial NO production. All 4 tissues eventually contract to A23187 due to the constant presence of adrenergic stimulation by NE and the smooth muscle calcium response to A23187. B, Data gathered as in A for the ability of aortic rings to relax on addition of 2-methylthio-ATP. C, Data gathered as in A for the A23187 response. Significance is indicated as **P < 0.01, ***P < 0.001. Data are representative of replicate observations in 3 independent experiments.
confers agonist-specific loss of receptor function is particularly relevant when viewed in the setting of endothelial dysfunction and cardiovascular disease.28–30

Cyclodextrins have been studied for some time as potential carriers for pharmaceuticals31 and their ability to incorporate cholesterol has proven to be of great utility. The specific role of β-mCD in the removal of plasmalemmal cholesterol has been well characterized.32 Cytotoxicity assays using different cyclodextrins demonstrate that β-mCD is nontoxic at concentrations higher and exposures longer than those used here. Exposure to 20 mmol/L β-mCD for 2 hours was shown to remove up to 90% of the cholesterol from mouse L cells without detectable adenine release.32 Our own results with cultured guinea pig endothelial cells show that exposure to 5 mmol/L for 20 minutes removes 54% of cholesterol measured by HPLC. Repletion of cholesterol with sterol-loaded cyclodextrins has been shown to re-establish inositol phosphate signaling in response to BK and EGF in systems where cholesterol was previously removed by empty cyclodextrin.16 This approach was ineffective in our studies (data not shown). Microscopic examination of control and β-mCD–treated aortic rings using hematoxylin-eosin staining (Figures 5A and 5B) demonstrates altered morphology of the endothelial lining in cyclodextrin-treated tissues. Control endothelium is seen as a contiguous monolayer across the basement membrane, whereas the cholesterol-depleted endothelium presents as discrete cells, partially withdrawn from each other. The apparent partial endothelial disruption notwithstanding, vital dye demonstrates that the endothelial cells in these β-mCD–treated tissues are viable (Figures 5B and 5C). Furthermore, detailed examination of the endothelium under electron microscopy reveals that caveolar structures are absent in β-mCD–treated tissues. These data are consistent with the notion that β-mCD renders the endothelium dysfunctional.

Despite the altered morphology of β-mCD–treated endothelium in guinea pig aorta, addition of A23187 first causes relaxation of vessel tone while vessels mechanically denuded of endothelium contract to A23187 (not shown). Thus, even with a dysfunctional endothelium, under these experimental conditions, one does not see immediate effects of A23187 directly on the smooth muscle. The subsequent contraction of all vessels to A23187 no matter their prior treatment confirms the action of the ionophore on the vascular smooth muscle and suggests probable depletion of endothelial NO after approximately 2 minutes. In control tissues where eNOS substrates have likely been depleted by prolonged NO signaling, or where eNOS is specifically inhibited by L-NAME pretreatment, the kinetics of contraction are rapid (Figure 7A). In aortic rings where P2Y receptor signaling to eNOS has been prevented by β-mCD or MRS2179 pretreatment, the apparent kinetics of contraction to A23187 are attenuated.

There is an interesting distinction between depletion of membrane cholesterol and removal of caveolin. The caveolin-1 knockout mouse demonstrates enhanced responsiveness to ACh over wild-type mice.29 In the present study, we report the complete absence of vascular relaxation in response to ACh in β-mCD–treated tissues. Using transmission electron microscopy, Drab et al29 demonstrate that endothelial cell caveolae are disrupted in caveolin-1 knockouts, eliminating the possibility that other caveolins can compensate for the loss. Taken together, these observations are consistent with the growing body of evidence that the association of caveolin-1 and eNOS inhibits NO formation.15,33 In caveolin-1 knockouts, eNOS cannot form an inhibitory complex with caveolin-1. This may allow increased NO production in response to ACh stimulation of contracted vessels. The direct association of receptor and eNOS is not likely given that eNOS activation is downstream of intracellular calcium release and not directly coupled to binding of ligand to receptor.

In our studies, eNOS is likely to be physically separated from receptor molecules in response to the disruption of caveolae (Figure 8). More importantly, the effect of β-mCD treatment uncouples receptor responses in a complex fashion. Although BK still causes the release of intracellular calcium in the endothelial cell, its ability to relax the vessel is lost. P2Y agonists on the other hand are neither able to raise intracellular calcium nor elicit relaxation. Such data suggest that signaling microdomains are able to respond to calcium activation of eNOS in a cholesterol-dependent fashion (Figure 8). These observations along with both the established inhibition of eNOS by caveolin and the activation of eNOS by calcium/calmodulin, lead to an intriguing hypothetical role for caveolae as receptor-specific calcium-sensing domains. It is possible to suggest that BK stimulation, unlike that of nucleotides, raises calcium without an effect of β-mCD treatment because in GPAECs the BK receptor is neither prelocalized to nor entirely dependent on the caveolar domain, whereas nucleotide receptors are. Support for this notion comes from studies showing that BK receptors move to cholesterol-rich signaling domains after stimulation.14,16 Despite such compartmentation of receptors, the β-mCD–treated cells still do not respond to BK-mediated increases in calcium with production of NO as might be expected if calcium has access to eNOS. The notion that agonist-specific compartmentation can extend beyond receptor localization is supported by the fact that global elevation of calcium, as occurs after A23187, produces endothelial-dependent relaxation not unlike controls. The large calcium elevation seen after BK stimulation of β-mCD–treated GPAECs (Figure 1B) does not equate to relaxation in β-mCD–treated tissues. Our data are consistent with the possibility that the caveolin-1:eNOS inhibitory complex cannot be overcome by agonist-mediated calcium elevation in the absence of cholesterol and/or cholesterol-dependent microdomain organization.

Nucleotide receptors must now be added to the list of signaling initiators associated with cholesterol-rich signaling microdomains. The association of these receptors with caveolae and/or DIGs suggests a potential paradigm for recognition of different nucleotides by endothelium under disparate conditions or anatomical locations. Endothelial cells may organize different P2Y receptors into different signaling domains in order to distinguish stimuli and regulate coupling to second messengers. When placed in the general context of local events occurring in blood vessels and the specific context offered by the Nucleotide Axis Hypothesis in particular, the regulation of nucleotide signaling offers increased...
understanding of vascular biology and possible future therapeutic targets for the treatment of cardiovascular disease.

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