Transport-dependent calcium signaling in spatially segregated cellular caveolar domains

Dihui Hong,1 Dov Jaron,1 Donald G. Buerk,2 and Kenneth A. Barbee1

1School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, Pennsylvania; and 2Departments of Physiology and Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania

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Hong D, Jaron D, Buerk DG, Barbee KA. Transport-dependent calcium signaling in spatially segregated cellular caveolar domains. Am J Physiol Cell Physiol 294: C856–C866, 2008. First published December 26, 2007; doi:10.1152/ajpcell.00278.2007.—We developed a two-dimensional model of transport-dependent intracellular calcium signaling in endothelial cells (ECs). Our purpose was to evaluate the effects of spatial colocalization of endothelial nitric oxide synthase (eNOS) and capacitative calcium entry (CCE) channels in caveolae on eNOS activation in response to ATP. Caveolae are specialized microdomains of the plasma membrane that contain a variety of signaling molecules to optimize their interactions and regulate their activity. In ECs, these molecules include CCE channels and eNOS. To achieve a quantitative understanding of the mechanisms of microdomain calcium signaling and the preferential sensitivity of eNOS to calcium entering the cell through CCE channels, we constructed a mathematical model incorporating the cell morphology and cellular physiological processes. The model predicts that the spatial segregation of calcium channels in ECs can create transport-dependent sharp gradients in calcium concentration within the cell. The calcium concentration gradient is affected by channel density and cell geometry. This transport-dependent calcium signaling specificity effect is enhanced in ECs by increasing the spatial segregation of the caveolar signaling domains. Our simulation significantly advances the understanding of how Ca2+ channels, despite its many potential actions, can mediate selective activation of signaling pathways. We show that diffusion-limited calcium transport allows functional compartmentalization of signaling pathways based on the spatial arrangements of Ca2+ sources and targets.

capacitative calcium entry; caveolae; endothelial cells; endothelial nitric oxide synthase; mathematical model

CALCIUM IS A UBQUITOUS MEDIATOR of intracellular signaling in virtually every cell type. A wide range of stimuli elicits changes in cytoplasmic calcium via influx through channels at the cell membrane or release from the endoplasmic reticulum (ER), and, yet, in many cases, the calcium changes appear to selectively activate different signaling pathways that are associated with the initial stimuli despite the similarity in the calcium changes themselves. Tymianski et al. (43) provided compelling evidence for this specificity in the case of glutamate excitotoxicity in spinal neurons. They found that even when intracellular calcium transients were of similar magnitude in response to a wide variety of stimuli, calcium entering the cytoplasm via the N-methyl-D-aspartic acid (NMDA) receptor channels preferentially leads to cell death. More recent work suggests NMDA receptor-mediated Ca2+ influx induces excitotoxic death via elevation in nuclear CCAAT enhancer binding protein (C/EBP-β) levels. In contrast, L channel-dependent Ca2+ influx results in decreased C/EBP-β level and improves neuron survival (14, 29). The identification of such distinct responses raises the question of how the same molecule can differentially activate diverse signaling pathways. The participation of calcium in a large number of physiological and pathological responses makes it an issue of wide reaching importance to understand how cells maintain specificity in calcium signaling despite its participation in many different signaling processes that may be activated in a cell.

One hypothesis is that Ca2+ channels and the signaling molecules associated with different pathways are nonuniformly distributed throughout the cell. The nonuniform distribution provides the mechanism by which cells are able to control Ca2+ entry at the right place, right time, and right concentration to initiate the appropriate signaling response. Such a mechanism would require spatial arrangement of channels and signaling molecules that permit large spatial gradients of calcium. Tymianski et al. (43) proposed that the signaling molecules initiating the pathway leading to cell death were physically linked or colocalized with the NMDA receptor channel (43). Another striking example of calcium specificity supporting the notion of spatial segregation of signaling pathways is the observation that while bulk average intracellular calcium concentration ([Ca2+]i) elevation in smooth muscle cells causes contraction, the local subplasma membrane calcium arising from ryanodine receptor activity promotes Ca2+-activated K+ channel opening to hyperpolarize the cell membrane and facilitate relaxation (31).

Numerous specialized domains in cells have been identified and found to contain concentrations of related signaling molecules, e.g., caveolae, lipid rafts, and focal adhesions. In domains like focal adhesions, there is clearly a need for spatial colocalization of the molecular constituents related to the organization of cell structure (e.g., to facilitate the assembly of actin bundles). However, one can also appreciate the potential for enhancement of biochemical reactions afforded by the spatial proximity of the myriad signaling molecules located there.

Caveolae are distinct flask-shaped invaginations of the plasma membrane. They are 50- to 100 nm in diameter and can exist individually or in clusters at the surface of the endothelium. Electron microscopy studies indicate that caveolae can exist luminally and abluminally, with the largest number in the peripheral region of the cell (34, 41). Caveolae have been suggested to be involved in signal transduction by containing signaling molecules, such as G protein and tyrosine kinase-
associated receptors, as well as the calcium-sensitive endothelial nitric oxide (NO) synthase (eNOS). Since Ca\(^{2+}\)-sensitive molecular effectors and cellular processes are compartmentalized in caveolae microdomains, it is reasonable to assume that the spatial and temporal organization of local calcium concentration in the microdomain is critical for proper cellular function. In the basal condition, caveolin-1, being the major coat protein of caveolae, binds to eNOS. This binding compromises the ability of eNOS to bind CaM, thereby inhibiting NO synthesis. Increases in \([Ca^{2+}]\) promote the binding of CaM to the CaM-binding motif, which is thought to displace an adjacent autoinhibitory loop on eNOS, thereby increasing eNOS activity.

In vascular endothelial cells (ECs), caveolae play an important role in the cell function by virtue of the colocalization of eNOS and its regulatory proteins, especially caveolin-1. ECs function to modulate vascular tone, regulate immune responses, control blood coagulation states, adjust vascular permeability, and prompt angiogenesis and vessel repair (42). Most of these endothelial functions depend on changes in \([Ca^{2+}]\). In response to vasoactive agonists (ATP, bradykinin, etc.) and mechanical stimuli, \([Ca^{2+}]\) is transiently elevated via release of Ca\(^{2+}\) from the ER through inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive channels. The depletion of intracellular Ca\(^{2+}\) stores is followed by Ca\(^{2+}\) influx via calcium channels in the cell membrane. This influx is referred to as capacitative calcium entry (CCE), also called store-operated calcium (SOC) entry. Transient receptor potential (TRP) proteins have been shown to form CCE channels via association either as homomers or heteromers with other TRPs (46). There is evidence that CCE channels reside in caveolae (21, 27) and that caveolar microdomains provide a scaffold for assembly and coordination of CCE signaling proteins (TRPs) into a complex (27).

eNOS has been shown to be more sensitive to CCE than to calcium elevation via ER release, and it has been proposed that colocalization of CCE calcium entry channels and eNOS in caveolae microdomains facilitates their interaction (22, 26). The cytosolic calcium diffusion coefficient \((D)\) is 250 \(\mu m^2/s\), suggesting that spatial segregation of calcium influx points on the microscale could produce significant gradients in calcium concentration. However, detailed calculation incorporating the various sources and sinks for calcium in specific geometric arrangements is needed to test whether the spatial proximity of CCE channels and eNOS could influence the calcium sensitivity of eNOS activation. Current experimental techniques are not capable of answering this question quantitatively. The most common technique used to reveal aspects of intracellular calcium signaling is the measurement \([Ca^{2+}]\), with a fluorescent calcium indicator. This method, however, has limited spatial and temporal resolution and is usually used to measure the average intracellular calcium changes. Recent advances in imaging techniques, such as confocal microscopy, provide a powerful tool to study the calcium dynamics in the micrometer range and have led to the report of microdomain calcium signals in a variety of excitable and nonexcitable cell types (21, 37). These “microdomains” have properties that vary in spatial dimensions (from 0.1 to few micrometers). The elementary events of Ca\(^{2+}\) signaling (Ca\(^{2+}\) blips, Ca\(^{2+}\) quarks, Ca\(^{2+}\) sparks, and Ca\(^{2+}\) puffs) appear to have a hierarchical organization that depends on the intensity of stimulus that triggers them. However, the quantitative characterization of the microdomain calcium signaling can only be predicted by mathematical models that incorporate interactions occurring at multiple length and time scales.

To achieve a quantitative understanding of these Ca\(^{2+}\) signaling mechanisms, the purpose of this work was to construct a model incorporating the morphology of the cell to predict the calcium response using our experimental data as well as data available in the literature. We show that modeling with realistic dimensions and geometry can provide a theoretical framework for analyzing a number of salient functional features that arise in the context of microdomain signaling. We investigate the geometric requirements (CCE channel localization and distribution) for the proposed mechanism of the selective sensitivity of eNOS to CCE. We further demonstrate how changes in cell morphology can affect transport-dependent calcium signaling.

**MATERIALS AND METHODS**

**Experimental Studies**

The detailed description of the cell culture for bovine aortic ECs (BAECs) and calcium measurement can be found in Hong et al. (17). For direct intracellular NO measurement, BAECs were loaded with 4-amino-5-methylamino-2′,7′-dichlorofluorescein (DAF-FM) diacetate (10 \(\mu M\)) for 30 min at room temperature. Changes in cellular DAF-FM fluorescence were normalized as \(F/F_0\), thus representing fold increases above basal level. All experiments were carried out at room temperature (22°–25°C).

**Caveolin-1 Immunocytochemical Staining**

Cells cultured on glass coverslips were washed three times with cold Dulbecco’s phosphate-buffered saline (DPBS). Then, the cells were fixed with 3% paraformaldehyde for 15 min and permeabilized in DPBS containing 0.1% Triton X-100 for 10 min at room temperature. Nonspecific binding sites were blocked by incubation with blocking solution (10% normal donkey serum in DPBS) for 30 min. Cells were then incubated at room temperature with primary antibody (anti-cav-1, BD Transduction) diluted in 1% donkey serum in DPBS to a final concentration of 1:200 for 1 h and with secondary antibody (anti-rabbit Alexa 488) diluted in 1% donkey serum in DPBS to a final concentration of 1:1,000 for 40 min in the dark.

**Chemicals and Reagents**

DPBS, nucloptides ATP, and EGTA were purchased from Sigma. Normal donkey serum was purchased from Jackson Immuno Research (West Grove, PA). The Fluoro-3-acetoxymethyl ester, DAF-FM, and anti-rabbit Alexa 488 were obtained from Molecular Probes. Tris-HCL buffer containing (in mM) 25 Tris-Tris-HCL, 137 NaCl, 2.7 KCl, 1 MgCl\(_2\), and 4 CaCl\(_2\) was used. The Ca\(^{2+}\)-free buffer consisted of Tris-HCL buffer solution in which CaCl\(_2\) was replaced with EGTA (1 mM).

**Mathematical Modeling**

Our model combines a simulation of cell morphology with cellular signaling. In particular, it focuses on the heterogeneous distribution of CCE channels on the cell membrane and provides quantitative analysis of how calcium concentration gradient is affected by channel density and cell geometry. The cell is modeled as a two-dimensional, disk-shaped object with a radius of 10 \(\mu m\) and a compartment representing the ER located in the center of the cell with a radius of 4.5 \(\mu m\), as shown in Fig. 1. The main parameters used in the simulations are defined in Table 1. The following physiological processes were included in the model: 1) diffusion of free Ca\(^{2+}\) in the cytoplasm; 2) binding of Ca\(^{2+}\) ions to an...
Calcium release model mediated by activation of the IP₃ receptor. The IP₃ receptor (IP₃R) open probability for IP₃-sensitive calcium channels (P_IP,R) in the ER is given by the following equation introduced by De Young and Keizer (11):

\[
P_{IP,R} = \frac{[Ca^{2+}]_{avg}[IP_3]}{[IP_3] + [IP_3]d_1 + d_2 + [Ca^{2+}]_{avg}d_3(\frac{[Ca^{2+}]_{avg}}{[Ca^{2+}]_{avg} + d_5})^2}
\]

in which \(d_1\), \(d_2\), \(d_3\), and \(d_5\) are the receptor dissociation constants for IP₃s, Ca²⁺ inhibition, IP₃s, and Ca²⁺ activation, respectively. This expression has been used by several other models (2, 24).

The outward flux of Ca²⁺ from ER (J_er) is determined by Ca²⁺ fluxes from IP₃R and passive leak:

\[
J_{er} = (v_{er}P_{IP,R} + v_{leak,er})([Ca^{2+}]_{er} - [Ca^{2+}]_{avg})
\]

where \(v_{er}\) is the maximal Ca²⁺ flux after IP₃R activation and \(v_{leak,er}\) is a leak flux constant.

Pump uptake. [Ca²⁺] is maintained by a combination of two calcium pumps: the SERCA pump and the PMCA pump. Ca²⁺ extrusion via the PMCA pump (J_PMCA) and uptake by SERCA pump (J_SERCA) are governed by Hill-type equations (28):

\[
J_{SERCA} = \frac{V_{max,SERCA}[Ca^{2+}]_{SERCA}}{[Ca^{2+}]_{avg} + K_{1/2,SERCA}}
\]

### Table 1. General Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R_{ec})</td>
<td>10 (\mu)m</td>
<td>Radius of EC</td>
</tr>
<tr>
<td>(R_{er})</td>
<td>4.5 (\mu)m</td>
<td>Radius of ER</td>
</tr>
<tr>
<td>(v_{er})</td>
<td>0.185</td>
<td>Volume ratio of ER to cytosol</td>
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<tr>
<td>(k)</td>
<td>0.7 (\mu)m</td>
<td>Dissociation constant for Ca²⁺</td>
</tr>
<tr>
<td>(d)</td>
<td>250 (\mu)m²/s</td>
<td>Diffusion coefficient of free Ca²⁺</td>
</tr>
<tr>
<td>(d_1)</td>
<td>0.5 (\mu)M</td>
<td>IP₃ dissociation</td>
</tr>
<tr>
<td>(d_2)</td>
<td>0.9 (\mu)M</td>
<td>Ca²⁺ inhibition</td>
</tr>
<tr>
<td>(d_3)</td>
<td>0.94 (\mu)M</td>
<td>IP₃ dissociation</td>
</tr>
<tr>
<td>(d_5)</td>
<td>0.082 (\mu)M</td>
<td>Ca²⁺ activation</td>
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<tr>
<td>(v_{IP₃})</td>
<td>3 (\mu)M/s</td>
<td>Maximum rate of IP₃ production</td>
</tr>
<tr>
<td>(v_{leak,er})</td>
<td>25 (\mu)M/s</td>
<td>Maximum Ca²⁺ permeability to ER</td>
</tr>
<tr>
<td>(v_{leak,er})</td>
<td>30 (\mu)M/s</td>
<td>Maximum Ca²⁺ permeability to ER</td>
</tr>
<tr>
<td>(V_{max,SERCA})</td>
<td>0.175 (\mu)M/s</td>
<td>Maximum flux across SERCA</td>
</tr>
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<td>(K_{1/2,SERCA})</td>
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<td>Maximum flux across PMCA</td>
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<td>(K_{1/2,PMCA})</td>
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<td>SERCA activation constant</td>
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<td>(K_{1/2,PMCA})</td>
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<td>PMCA activation constant</td>
</tr>
<tr>
<td>(R_{PMCA})</td>
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<td>SERCA Hill coefficient</td>
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<tr>
<td>(R_{PMCA})</td>
<td>2</td>
<td>PMCA Hill coefficient</td>
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<td>(k_{on})</td>
<td>100 (\mu)M/s</td>
<td>On-rate of nondiffusible buffer</td>
</tr>
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<td>(k_{off})</td>
<td>500 (s^{-1})</td>
<td>Off-rate of nondiffusible buffer</td>
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<td>(I_{soc})</td>
<td>0.11 (s^{-1})</td>
<td>CIF degradation constant</td>
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<tr>
<td>(v_{CIF})</td>
<td>0.078 (s^{-1})</td>
<td>CIF permeability across the ER</td>
</tr>
<tr>
<td>(V_{CIF})</td>
<td>0.018 (s^{-1})</td>
<td>CIF production rate</td>
</tr>
<tr>
<td>(v_{soc})</td>
<td>0.035 s/(\mu)M</td>
<td>SERCA permeability, (\mu)M</td>
</tr>
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</table>

**EC.** endothelial cell; ER, endoplasmic reticulum; IP₃, inositol 1,4,5-trisphosphate; SERCA, sarco(endo)plasmic reticulum Ca²⁺-ATPase; PMCA, plasma membrane Ca²⁺-ATPase; SOC, store-operated calcium; CIF, calcium influx factor; CCE, capacitative calcium entry.

![Model geometry and the processes included in the model. CCE, capacitative calcium entry; CIF, calcium influx factor; J_SERCA, Ca²⁺ uptake by the sarc(endo)plasmic reticulum Ca²⁺-ATPase pump; J_PMCA, Ca²⁺ extrusion via the plasma membrane Ca²⁺-ATPase pump; Ca_sent, cytosolic calcium; Ca₂⁺, extracellular calcium concentration; PIP₂, phosphatidylinositol bisphosphate; PLC, phospholipase C.](image-url)
where $V_{\text{max}}$ is maximum flux.

**Calcium buffering.** The rate of change of free [Ca$^{2+}$] is dependent on the reversible binding with soluble buffering proteins (45). The buffering proteins are considered immobile (nondiffusible) and uniform throughout the cytosol. The total concentration of calcium binding sites is denoted by $B_T$. $[B]$ and $[CaB]$ are the concentrations of the unbound and bound forms of the buffer present. $k_{\text{off}}$ and $k_{\text{on}}$ are the reverse and forward rate constants, respectively, of the binding reaction. The rate at which calcium binds to proteins is proportional to the free calcium concentration and the concentration of free binding sites. Calcium also dissociates from the protein at a rate proportional to the concentration of the complex. Thus the rate of free calcium change due to the protein buffering is represented by:

$$
\frac{d[C_{\text{un}}]}{dt} = -k_{\text{off}}[CaB] + k_{\text{on}}[Ca^{2+}][B_T] - [Ca_{\text{un}}]
$$

(7)

The initial condition for buffered calcium $C_{\text{un}}$ can be calculated by setting Eq. 7 to zero: all other initial values used are listed in Table 2.

**CCE model.** A signal generated by depletion of intracellular calcium stores activates CCE. The signal transduction mechanism linking changes in intraluminal Ca$^{2+}$ to the opening of plasma membrane Ca$^{2+}$ channels is still controversial. A putative calcium influx factor (CIF) was proposed to act as a second messenger in ECs (32, 42). When ER concentration of [Ca$^{2+}$] decreases below a certain level, CIF is rapidly produced and released from ER, activating CCE entry.

$$
\frac{d[CIF]_{\text{cyt}}}{dt} = -v_{\text{CIF}}([CIF]_{\text{cyt}} - [CIF]_{\text{cyt,0}}) + k_{\text{CIF}}([CIF]_{\text{cyt,0}} - [CIF]_{\text{cyt}})
$$

(8)

$$
\frac{d[CIF]_{\text{ER}}}{dt} = r_{\text{CIF}}v_{\text{CIF}}([CIF]_{\text{cyt}} - [CIF]_{\text{ER}}) - k_{\text{off}}[CIF]_{\text{ER}}
$$

(9)

The flux of calcium through CCE channels ($J_{\text{CCE}}$) is a function of cytosolic CIF concentration and depends on the level of extracellular calcium. The mathematical expression to describe this behavior is given by:

$$
J_{\text{CCE}} = v_{\text{CCE}}[CIF]_{\text{cyt,0}}[Ca^{2+}]_{\text{cyt}} - [Ca^{2+}]_{\text{ER}}
$$

(10)

where $v_{\text{CCE}}$ denotes the calcium permeability of CCE channels. CCE channels are nonuniformly distributed on the cell membrane, and the CCE influx processes are present only in a segment of the cell membrane where CCE channels are located.

**Final model.** Combining Eqs. 1–10 yields the differential equation for [Ca$^{2+}$]:

$$
\frac{d[Ca]_{\text{cyt}}}{dt} = D\nabla^2[Ca^{2+}] + k_{\text{on}}[CaB] - k_{\text{off}}[Ca^{2+}][B_T] - [Ca_{\text{un}}] + J_{\text{IP,R}} + J_{\text{SERCA}} + J_{\text{CCE}} - J_{\text{PMCA}}
$$

(11)

### Table 2. Initial concentration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ca$^{2+}$]$_{\text{cyt}}$</td>
<td>0.1 μM</td>
<td>Cytosolic calcium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_{\text{ER}}$</td>
<td>0.1 μM</td>
<td>Spatial average cytosolic calcium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_{\text{ER}}$</td>
<td>350 μM</td>
<td>Calcium concentration in ER</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_{\text{ER}}$</td>
<td>4 mM</td>
<td>Extracellular calcium concentration</td>
</tr>
<tr>
<td>[IP$<em>3$]$</em>{\text{ex}}$</td>
<td>0.03 μM</td>
<td>Cytosolic IP$_3$ concentration</td>
</tr>
<tr>
<td>[IP$<em>3$]$</em>{\text{cyt}}$</td>
<td>20 μM</td>
<td>ER IP$_3$ concentration</td>
</tr>
<tr>
<td>[IP$<em>3$]$</em>{\text{cyt}}$</td>
<td>0 μM</td>
<td>Cytosolic IP$_3$ concentration</td>
</tr>
</tbody>
</table>

The change over time of the calcium concentration in ER ([Ca$^{2+}$]$_{\text{ER}}$) and in the extracellular medium ([Ca$^{2+}$]$_{\text{ex}}$) is described by:

$$
\frac{d}{dt}[Ca^{2+}]_{\text{ER}} = (-J_{\text{IP,R}} + J_{\text{SERCA}})_{\text{er}}
$$

(12)

$$
\frac{d}{dt}[Ca^{2+}]_{\text{ex}} = 0
$$

(13)

The set of coupled nonlinear partial differential equations written for the model were solved by the finite element method using commercial software (FlexPDE 3; PDESolutions, Antioch, CA). The mesh densities were adaptively refined by the program to ensure a relative accuracy of 0.0001 for the numerical solutions.

**RESULTS**

**Experimental Results.**

ATP-induced calcium release from ER and CCE. In our experiments, Fluo-3 was used to assess global [Ca$^{2+}$], in response to ATP in ECs, and fluorescence was expressed in normalized units ($F/F_0$). The application of ATP to BAECs in the presence of extracellular Ca$^{2+}$ elicits a Ca$^{2+}$ response that is a combination of ER release and CCE. To separate ER Ca$^{2+}$ release from CCE, cells were first exposed to 50 μM ATP in the absence of external Ca$^{2+}$, thus preventing the CCE. Treatment with ATP led to a transient increase in [Ca$^{2+}$], owing to calcium release from ER, reaching a peak after 5–10 s and returning to the basal level within 1–2 min (depicted in the left portion of Fig. 2). Subsequent exposure to 4 mM extracellular Ca$^{2+}$ resulted in a rapid and sustained [Ca$^{2+}$] elevation (depicted in the right portion of Fig. 2), reflecting mostly Ca$^{2+}$ influx through CCE pathway since CCE is the predominant source of agonist-regulated Ca$^{2+}$ entry into ECs (42). Compared with the IP$_3$-mediated ER release, capacitative entry of calcium increases plateau level but has lower peak amplitude.

**Effect of ER Ca$^{2+}$ release and CCE on NO production.** For direct intracellular NO measurement, BAECs were loaded with membrane-permeable DAF-FM diacetate. NO does not dissociate from DAF-FM once this dye reacts with NO. Thus the NO-sensitive fluorescence with DAF primarily represents a cumulative concentration of NO within the cells. This can be represented by the following equation:

$$
\frac{d[\text{DAF}]}{dt} = k_{\text{off}}[\text{NO}][\text{DAF}] - k_{\text{on}}[\text{NODAF}]
$$

The right-hand term $k_{\text{off}}$ is zero, and [DAF] is much larger than [NO] and thus can be considered as constant. Therefore, the concentration of NO is proportional to the time derivative of the fluorescence intensity $[d(F/F_0)/dt]$. Stimulation of BAECs with ATP in calcium-free medium caused only a slight increase in NO production $[d(F/F_0)/dt] = 0.0239\text{ min}^{-1}$ in the first 3 min (Fig. 3) despite the large Ca$^{2+}$ transient (Fig. 2). In contrast, subsequent addition of 4 mM Ca$^{2+}$ caused a large and sustained increase in NO production detected during CCE entry $[d(F/F_0)/dt] = 0.0819\text{ min}^{-1}$, even though the peak of calcium magnitude from calcium entry was significantly lower than from ER calcium release. Bredt and Snyder (7) demonstrated that within the physiological range of calcium concentration (0.1–5 mM), the activity of isolated eNOS increases linearly with Ca concentration. Thus, the cumulative produc-
tion of NO over a period of time should depend on the calcium signal integrated over that time rather than on the magnitude of calcium peak alone. To allow for the fact that NO release is also a function of Ca\(^{2+}\) elevation duration, we also determined total Ca\(^{2+}\) load by integrating the spatial average \([\text{Ca}\(^{2+}\)]_i\) over time (3 min). In the first 3 min after addition of extracellular calcium, the integrated calcium load was only 1.425 times the load due to the transient release for the ER. Nevertheless, the CCE-induced NO production indicated by \(d(F/F_0)/dt\) was 3.43 times that induced by ER calcium release.

**Distribution of caveolae on EC membrane.** BAECs were immunostained with polyclonal antibody to caveolin-1. Caveolin-1 was distributed heterogeneously in the cells and tended to display a typical clustered pattern at specific parts of the cell, preferentially the peripheral region of the cell as shown in Fig. 4.

**Simulation Results**

**Simulation of calcium response to ATP in the absence of external Ca\(^{2+}\).** The effect of ATP-induced IP\(_3\)-mediated calcium release from ER in ECs with calcium-free medium was modeled for a disk-shaped cell. In the absence of external Ca\(^{2+}\), ATP causes a significant depletion of calcium in the ER calcium stores, and there is no calcium influx from CCE channels. Free calcium diffuses in the cytosol, binds with buffering proteins, and is extruded via the PMCA pumps and SERCA pumps. The calculated time course of calcium changes for \([\text{Ca}\(^{2+}\)]_m\) (10 nm from the cell membrane) and the spatial average of intracellular calcium changes \([\text{Ca}\(^{2+}\)]_\text{avg}\) are nearly identical, and both are similar to the experimental observations (Fig. 5A). The spatial distribution of \([\text{Ca}\(^{2+}\)]_i\) after 10 s of ER calcium release (Fig. 5B) indicates that when calcium is released uniformly from the ER, very small calcium gradients exist inside the cell. The initial increase in \([\text{Ca}\(^{2+}\)]_i\); at 10, 1,000, 2,000, 3,000, and 4,000 nm from the membrane is plotted on an expanded time scale showing that the small spatial gradient dissipated within 5 s (Fig. 5C).

**Simulation of CCE.** The depletion of intracellular Ca\(^{2+}\) stores is followed by Ca\(^{2+}\) influx through CCE channels, which are nonuniformly distributed on cell membrane (Fig. 4). In our model, we assumed two 180-nm segments of the membrane in which CCE channels are clustered. Our model predicts a large discrepancy between \([\text{Ca}\(^{2+}\)]_m\) (calcium concentration at a point in the cytosol, 10 nm from the membrane in the center of the cell membrane segment containing the CCE channels domain) and \([\text{Ca}\(^{2+}\)]_\text{avg}\) (Fig. 6A). A spatial map of \([\text{Ca}\(^{2+}\)]_m\); after 30 s CCE indicates large calcium gradients near the clustered CCE channels (Fig. 6B). Profiles of \([\text{Ca}\(^{2+}\)]_i\); at positions 10, 1,000, 2,000, 3,000, and 4,000 nm from the membrane show the dependence of the calcium signal on proximity to the CCE-rich domain.

**Influence of CCE channels distribution on calcium gradient.** To investigate the CCE channels/caveolae clustering on the calcium dynamics, we performed a parametric analysis of the CCE channels density (Fig. 7). In our simulation, we varied...
the CCE distribution by changing the span of the membrane segment occupied by the CCE channels domain. The total calcium flux from the CCE channels was kept constant by making the value of CCE permeability inversely proportional to the length of CCE domain. This is equivalent to

**Fig. 5.** Simulation of IP3-mediated calcium release from ER. **A:** comparison of localized calcium concentration 10 nm from cell membrane ([Ca2+]m) and the spatial average of intracellular calcium concentration ([Ca2+]avg). Also plotted for comparison is an experimental (exp) calcium transient. **B:** color map of the distribution of [Ca2+]i after 10 s ER calcium release (spatial dimensions in μm). **C:** traces of free [Ca2+] (located at 10, 1,000, 2,000, 3,000, and 4,000 nm underneath the membrane) versus time. For spatially uniform release of calcium from the ER, gradients are dissipated by diffusion in <5 s (see inset).

**Fig. 6.** Simulation of CCE entry. **A:** large discrepancy between submembranous calcium levels in caveolae microdomain ([Ca2+]m) and [Ca2+]avg is predicted by simulation. **B:** color map of the distribution of [Ca2+]i, after 30 s CCE entry (spatial dimensions in μm). **C:** profile of [Ca2+]i at depth of 10, 1,000, 2,000, 3,000, and 4,000 nm from cell membrane. Peak calcium concentration at membrane is significantly higher than in the bulk cytoplasm.
keeping the number of CCE channels constant while spreading them over different areas. As the CCE channels become less localized, the cytoplasmic calcium becomes more uniform, and no sharp gradients appear. Increasing the cluster density of CCE channels significantly increased the \([Ca^{2+}]_{\text{m}}\) (Fig. 7A) without significantly changing the spatially averaged calcium \([Ca^{2+}]_{\text{avg}}\) (Fig. 7B).

**Effect of cell geometry on calcium gradient.** The effects described above are for a round cell. In vivo ECs in straight arterial segments and in vitro monolayers exposed to flow become elongated and aligned with the direction of flow. The width-to-length ratio of the elongated ECs cells has been found to be in the range of 0.2–0.29 (10). We investigated the potential functional consequences of further segregating the CCE domains as the cells elongate in response to flow. We simulated the calcium response in elliptically shaped cells, keeping the number of CCE channels constant while spreading them over different areas. As the CCE channels become less localized, the cytoplasmic calcium becomes more uniform, and no sharp gradients appear. Increasing the cluster density of CCE channels significantly increased the \([Ca^{2+}]_{\text{m}}\) (Fig. 7A) without significantly changing the spatially averaged calcium \([Ca^{2+}]_{\text{avg}}\) (Fig. 7B).

**Effect of ER location on calcium gradient.** Besides the CIF model, another theory—the conformational coupling model—has been proposed to explain the signaling mechanism between intracellular Ca\(^{2+}\) stores and CCE. This model suggests ER and CCE may be physically close to one another and that there is a direct protein-protein interaction between the ER and CCE channel in the membrane. The IP\(_3\)-Rs are hypothesized to play a role in this signaling by using their large cytoplasmic heads to transmit the information. Because this mechanism requires the ER to be in close proximity to the plasma membrane, we performed simulations to examine the effect of the separation distance between the ER and CCE channels (Fig. 9). When the ER membrane is 500 nm from the portion of cell membrane where CCE channels reside, there is an even greater enhancement of the local calcium concentration near the CCE domain (Fig. 9A). As the separation distance between the ER and the CCE domain increases, the calcium concentration near the CCE channels decreases dramatically (Fig. 9B).

**DISCUSSION**

Calcium is a ubiquitous second messenger linking a variety of external stimuli to cellular responses. Ca\(^{2+}\) enters the cell through several kinds of calcium channels and has the ability to selectively couple to different physiological processes. Ca\(^{2+}\) signaling inherently involves three factors: time, space, and amplitude. The correct spatial and temporal control of calcium signaling is essential for this targeted signaling (5).

A number of mathematical models for calcium transients have been published for different cell types, including ECs. These models of cellular calcium dynamics have traditionally been of a lumped-parameter type, i.e., systems of ordinary differential equations (2, 11, 45). The use of such formalism assumes no spatial gradients in the calcium concentration. There are some studies that explore calcium distribution throughout a model cell. These studies mainly focus on the impact of pulse duration, buffer diffusion rates, buffer affinity, and kinetics on the shape of the calcium transients (23, 25, 30, 33, 38). Modern imaging techniques have recently shown that spatially organized Ca\(^{2+}\) waves originate in subsets of clustered caveolae regions at the edges of ECs in response to IP\(_3\)-mobilizing agonist ATP (18–20). In the research reported here, we have highlighted the importance of the spatial segregation of CCE channels—leading to different concentration gradients within the cell—in determining the targeted calcium signaling, especially NO signaling.

eNOS, a calcium-dependent enzyme, is dually acylated by the fatty acids myristate and palmitate, and these modifications target eNOS to plasma membrane caveolae domains (13, 15). The proper targeting of eNOS to caveolae has been suggested as a prerequisite for effective enzyme activation. Mislocalization of eNOS in the cytoplasm by oxidized LDL treatment, cholesterol depletion, or acylation-deficient mutation reduces both the basal and the stimulated NO production (6, 9, 22, 40). Furthermore, Jagnandan et al. (22) compared the activity of inducible (iNOS) and eNOS after targeting them to different subcellular locations, and, in contrast with eNOS, no impairment in the ability of iNOS to synthesize NO was found. iNOS is structurally similar to eNOS and has virtually identical

cofactor and substrate affinities with one exception—calcium dependency. eNOS is highly dependent on increases in intracellular \( \text{Ca}^{2+} \) for activity, whereas iNOS is \( \text{Ca}^{2+} \) independent. Therefore, it has been postulated that targeting of eNOS to caveolae domains causes eNOS to experience higher calcium concentrations due to the colocalization of eNOS and CCE channels. Thus, the production of steep calcium gradients represents a plausible explanation for the caveola localization-dependent activity of eNOS (22).

Several recent studies suggest that CCE is more effective in the activation of eNOS, while other means of elevating \( \text{Ca}^{2+} \), cofactor and substrate affinities with one exception—calcium dependency. eNOS is highly dependent on increases in intracellular \( \text{Ca}^{2+} \) for activity, whereas iNOS is \( \text{Ca}^{2+} \) independent. Therefore, it has been postulated that targeting of eNOS to caveolae domains causes eNOS to experience higher calcium concentrations due to the colocalization of eNOS and CCE channels. Thus, the production of steep calcium gradients represents a plausible explanation for the caveola localization-dependent activity of eNOS (22).

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Fig. 8. Comparison of ellipses with different eccentricity. A: color maps of the distribution of \( \text{[Ca}^{2+}] \), after 30 s CCE entry (spatial dimensions in \( \mu \text{m} \)). The ER membrane is 500 nm from the portion of cell membrane where CCE channels reside. B: profiles of calcium transients positioned 10 nm underneath the membrane in the caveolae microdomain. Different profiles correspond to different location of ER. ER membrane is 0.5, 1, 2, and 5.5 \( \mu \text{m} \) underneath the cell membrane, respectively. Proximity of the ER to the plasma membrane enhances the peak \( \text{[Ca}^{2+}] \) by reducing the space available for diffusion.

Fig. 9. Effect of ER localization on calcium signaling. A: color map of the distribution of \( \text{[Ca}^{2+}] \), after 30 s CCE entry (spatial dimensions in \( \mu \text{m} \)). The ER membrane is 500 nm from the portion of cell membrane where CCE channels reside. B: profiles of calcium transients positioned 10 nm underneath the membrane in the caveolae microdomain. Different profiles correspond to different location of ER. ER membrane is 0.5, 1, 2, and 5.5 \( \mu \text{m} \) underneath the cell membrane, respectively. Proximity of the ER to the plasma membrane enhances the peak \( \text{[Ca}^{2+}] \) by reducing the space available for diffusion.
are ineffective (22, 26). Our experimental results are consistent with those studies, and our simulations provide support for the hypothesis that the mechanism for this selectivity is related to calcium transport. Furthermore, the results of our simulation indicate the spatiotemporal profile of intracellular calcium signals depends heavily on the spatial arrangement of calcium channels. If Ca\(^{2+}\) is released uniformly from ER, or if CCE channels were distributed uniformly over the cell membrane, there would be no significant differences between the average calcium concentration and the locally elevated concentration in the subplasmalemma domain. Thus, the differential sensitivity of eNOS to CCE calcium requires both the clustering of CCE channels and the colocalization of eNOS. The study by Lin et al. (26) provides evidence to support our simulation results. They observed that ionomycin, which elevates [Ca\(^{2+}\)], by facilitating uniform, nonlocalized transmembrane calcium influxes, produces a comparable increase in average intracellular concentration to thapsigargin in the presence of extracellular calcium. However, the increase in NO production is much lower than that produced by thapsigargin-stimulated CCE.

The caveolin-1 immunostaining result (Fig. 4) shows the heterogeneous distribution of caveolae domain in the ECs. The picture clearly indicates that caveolae tend to accumulate at the edge of ECs, an observation consistent with previous reports (8, 15, 19, 41). Quantified by electron microscopy, the caveolae number in endothelium in vivo is observed to be much higher than ECs in culture (34, 41). The greater influx due to the higher number of caveolae would only increase the gradients in calcium, so the difference in caveolae number does not affect the conclusions from our simulations of the in vitro response. As shown in Fig. 6, clusters of CCE channels can contribute to the buildup of high calcium concentration gradients, and these gradients are determined by channel cluster density. Higher cluster density allowed for higher microdomain calcium concentrations. We also examined the effect of close proximity of ER to CCE domains on the localized calcium concentration. Despite the close proximity of the calcium-pumping activity of the ER membrane, the decreased volume of cytoplasm created by the presence of this organelle hinders the dissipation of the calcium gradient by diffusion into the cytoplasm. The calcium buffer in the vicinity of CCE channels gets locally saturated, resulting in the buildup of a high calcium concentration domain. We have modeled the cell as having a single large organelle surrounded by a large cytoplasmic volume. The inclusion of other organelles would further reduce the cytoplasmic volume available for diffusive transport of calcium. Thus, similar to the effect of moving the ER compartment close to the plasma membrane, the incorporation of additional organelles distributed throughout the cytoplasm would tend to enhance the gradients in calcium.

Under shear stress, ECs become elongated and polarized. Isshiki et al. (19) observed that exposing ECs to shear stress caused polarization of caveolae on cell surface and relocated the caveolin-1 to the upstream edge of the cell (19). At the same time, CCE channels also were repositioned at the upstream edge of the cells. Our results suggest that polarization of the CCE channels, concomitant with relocation of the caveolae, increases the calcium gradient in the microdomain, suggesting a novel mechanism by which the well-known flow-induced elongation of ECs can affect their signaling behavior. Flow-induced alignment of ECs reduces the peak shear stress and shear stress gradients experienced by the cells, suggesting the aligned cells may be less sensitive to shear stress than nonaligned cells at a given flow rate (3, 4). In contrast, the enhancement of the calcium transport effects in elongated cells provides a mechanism for increased sensitivity to shear stress consistent with the findings of Rizzo et al. (36). They showed that, in flow-conditioned ECs, a step increase in shear stress significantly enhanced tyrosine phosphorylation of luminal surface proteins, including caveolin-1, and Ser\(^{179}\) phosphorylation of eNOS compared with static culture cells (1). The shear stress-mediated Cas tyrosine phosphorylation has been shown to be calcium dependent (35). Another implication of our results is that, in elongated ECs, high calcium concentrations in the caveolae domains can be achieved even when the spatial average concentration is relatively low. This may explain why, in intact vessels, NO-mediated dilation is observed in response to increases in shear stress despite only minor calcium changes (44). In our experimental results, the integrated calcium load ([Ca\(^{2+}\)] \(_{avg}\) of CCE is 1.42 times that due to ER release. However, the CCE-induced NO production indicated by \(d(F/F_{0})/dt\) is 3.43 times that induced by ER calcium release. Assuming NO production is proportional to the calcium concentration as suggested by Bredt and Snyder (7), we can predict the NO production based on the calcium concentration at the caveolar domain (where eNOS resides). Our simulation results showed that integrated calcium concentration in the caveolar domain can be up to three times the average [Ca\(^{2+}\)], (depending on channel density, cell shape, etc.), consistent with the observed NO production due to CCE.

The results of our model are generally in good agreement with experimental data. However, as with all models, ours is limited by certain assumptions. Some of our assumptions have minor effects on the predictions of our model; others will have to be examined in future models. First, in this model, it is assumed that the calcium diffusion coefficient is homogeneous through the whole cell. Naraghi et al. (30) revealed the inhomogeneity of calcium diffusion coefficient in chromaffin cells. Their data indicated that the Ca\(^{2+}\) diffusivity is lowest under the plasma membrane. Nevertheless, the inhomogeneity of calcium diffusivity will not affect our conclusions since the restricted diffusion at the cell membrane should enhance the calcium gradient in the caveolae–clustered microdomain.

Second, we assumed the buffering protein concentration to be homogeneous. Naraghi et al. (30) performed photolysis experiments and did not observe significant variation of calcium buffer distribution on a micrometer spatial scale in bovine chromaffin cells. We only considered the fixed buffer and ignored the mobile buffer. We reasoned that the mobile buffer concentration is relatively low, and even when mobile buffers are considered, the diffusion coefficient of mobile buffer will be much lower than calcium diffusion coefficient. Sala and Hernandez-Cruz (38) showed that the mobility of the buffers has little influence on the calcium transients in the outermost shells of the cells. We also ignored the calcium extrusion by the Na\(^+/Ca\(^{2+}\) exchanger (NCX) on the cell membrane and only considered the PMCA pump since there is evidence that PMCA is important for Ca\(^{2+}\) extrusion while NCX plays a minor role (39). We performed a simulation that included the contribution of the NCX to [Ca\(^{2+}\)] extrusion and found that the average calcium concentration was not significantly affected, nor was the calcium gradient in the caveolae domain.

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We also neglected the effects of mitochondrial sequestering, which, because of its low affinity, was not expected to influence the results for the transient response we examined (16, 42). To verify this, an additional simulation was performed in which mitochondria were introduced as a cluster near (1 μm) the CCE microdomain. We used the model of mitochondria calcium signaling described by Falcke et al. (12), which includes calcium uptake via a mitochondrial Ca\(^{2+}\) uniporter and calcium efflux through Ca\(^{2+}/\text{Na}^+\) pathway. As expected, we found that the presence of mitochondria did not significantly affect the calcium concentration.

Finally, in the present study, we did not consider the IP\(_3\) and CIF diffusion and the impact on the calcium signaling. We used disk-shaped cell rather than real cell geometry to calculate the spatial and temporal distribution of calcium. These have to be included in future models because the cell geometry also affects IP\(_3\) and CIF diffusion, and thus it influences calcium signaling. In addition, we assumed the CCE channels to be distributed uniformly across the whole caveolae-clustered domain and ignored the spatial arrangement of individual caveolae within the domain. If the calcium channel properties in a single caveola are known, our model can be further extended to calculate the calcium profile in neighborhood of a single caveola and determine how much separation between caveolae would be necessary to produce even smaller scale gradients in calcium. Despite these limitations, our model adequately reproduces the experimental results and provides important insights into the role of the spatial arrangement of CCE channels in shaping the calcium gradient and to what extent this gradient is influenced by the cluster density of CCE channels and the cell geometry. Our results indicate that the clustering of calcium channels results in steep microdomain calcium gradients. Our simulations support the idea that functional coupling between Ca\(^{2+}\) channels and Ca\(^{2+}\)-regulated targets can be achieved through spatial cocolocalization of the source and target and through spatial segregation from other sources. We provide a specific example of transport-dependent signaling specificity that provides functional compartmentalization of a signaling pathway that could be generalized to model other signaling molecules and pathways.

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