**Na⁺/Ca²⁺ Exchange Facilitates Ca²⁺-dependent Activation of Endothelial Nitric-oxide Synthase**

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Recent evidence suggests the expression of a Na⁺/Ca²⁺ exchanger (NCX) in vascular endothelial cells. To elucidate the functional role of endothelial NCX, we studied Ca²⁺ signaling and Ca²⁺-dependent activation of endothelial nitric-oxide synthase (eNOS) at normal, physiological Na⁺ gradients and after loading of endothelial cells with Na⁺ ions using the ionophore monensin. Monensin-induced Na⁺ loading markedly reduced Ca²⁺ entry and, thus, steady-state levels of intracellular free Ca²⁺ ([Ca²⁺]i) in thapsigargin-stimulated endothelial cells due to membrane depolarization. Despite this reduction of overall [Ca²⁺]i, Ca²⁺-dependent activation of eNOS was facilitated as indicated by a pronounced leftward shift of the Ca²⁺ concentration response curve in monensin-treated cells. This facilitation of Ca²⁺-dependent activation of eNOS was strictly dependent on the presence of Na⁺ ions during treatment of the cells with monensin. Na⁺-induced facilitation of eNOS activation was not due to a direct effect of Na⁺ ions on the Ca²⁺ sensitivity of the enzyme. Moreover, the effect of Na⁺ was not related to Na⁺ entry-induced membrane depolarization or suppression of Ca²⁺ entry, since neither elevation of extracellular K⁺ nor the Ca²⁺ entry blocker 1-(β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenethyl)-1H-imidazole hydrochloride (SK&F 96365) mimicked the effects of Na⁺ loading. The effects of monensin were completely blocked by 3',4'-dichlorobenzamil, a potent and selective inhibitor of NCX, whereas the structural analog amiloride, which barely affects Na⁺/Ca²⁺ exchange, was ineffective. Consistent with a pivotal role of Na⁺/Ca²⁺ exchange in Ca²⁺-dependent activation of eNOS, an NCX protein was detected in caveolin-rich membrane fractions containing both eNOS and caveolin-1. These results demonstrate for the first time a crucial role of cellular Na⁺ gradients in regulation of eNOS activity and suggest that a tight functional interaction between endothelial NCX and eNOS may take place in caveolae.

The endothelial isoform of nitric-oxide synthase (eNOS) is constitutively expressed in endothelial cells and cardiac myocytes and dynamically regulated by Ca²⁺/calmodulin. The enzyme is unique among the three known NOS isoforms in being targeted to specialized cell surface signal-transducing domains termed plasmalemmal caveolae (1, 2). This feature appears to allow for an efficient control of eNOS activity, as numerous signaling molecules such as G-protein-coupled receptors, the plasma membrane Ca²⁺ pump, an inositol 1,4,5-trisphosphate-sensitive Ca²⁺ channel, and protein kinase C are enriched in caveolae (3, 4). The targeting of eNOS to caveolae is promoted by direct interaction of the enzyme with the caveolae structural protein, caveolin-1. The interaction of eNOS with caveolin-1 renders the enzyme inactive, apparently due to a functional competition between caveolin-1 and Ca²⁺/calmodulin (5–7). In the presence of high concentrations of Ca²⁺, the NOS-caveolin complex dissociates, and a catalytically active NOS-Ca²⁺/calmodulin complex is formed.

Although it is well established that depletion of intracellular Ca²⁺ stores and capacitative Ca²⁺ entry across the endothelial plasma membrane are key events in endothelial Ca²⁺ signaling (8–10), little is known about the role of individual Ca²⁺ transport systems in eNOS regulation. Recent studies provide evidence that significant increases in subplasmalemmal [Ca²⁺i], which may well be sufficient for enzyme activation, may occur even in the absence of detectable changes in perinuclear [Ca²⁺i], suggesting that focal elevations in subplasmalemmal [Ca²⁺i], rather than increases in overall [Ca²⁺i], trigger NO biosynthesis in endothelial cells (11, 12). Thus, the subcellular Ca²⁺ distribution and the subplasmalemmal Ca²⁺ concentration at the caveolae may be of particular importance for modulation of eNOS activity. It has recently been postulated that endothelial subplasmalemmal [Ca²⁺i], may be controlled for a large part by Na⁺/Ca²⁺ exchange (11). This Ca²⁺ transport system was first identified in cardiac muscle (13) and transports Ca²⁺ in exchange for Na⁺ in either direction, depending on the electrochemical gradients of Na⁺ and Ca²⁺ (14, 15). In the forward mode (Na⁺ entry/Ca²⁺ extrusion), the exchanger represents the primary mechanism for Ca²⁺ efflux in the myocardium and thus plays a prominent role in contractile function (15–17). During depolarization, the exchanger operates in reversed mode (Ca²⁺ entry/Na⁺ extrusion) and triggers Ca²⁺-induced Ca²⁺ release during cardiac excitation (18, 19).

In endothelial cells, the presence of a Na⁺/Ca²⁺ exchanger (NCX) has recently been demonstrated by immunoblotting and immunofluorescence microscopy (20), and these results have been verified by the detection of cDNA coding for NCX1 (21). However, the role of this protein in endothelial Ca²⁺ homeostasis is still a matter of debate. Since inhibition of NCX diminished the endothelium-dependent relaxation of arteries (22, 23), it has been suggested that reversed mode Na⁺/Ca²⁺ exchange may contribute to the plateau phase of [Ca²⁺i], after agonist stimulation of endothelial cells (24). However, no
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change in overall [Ca²⁺], was observed when extracellular Na⁺ was replaced by N-methyl-D-glucamine or Li⁺ in resting or bradykinin-stimulated endothelial cells (25, 26). Nonetheless, a significant increase in [Ca²⁺], was observed when extracellular Na⁺ was replaced by Li⁺ in endothelial cells that were Na⁺-loaded by a preceding incubation with the Na⁺ ionophore monensin (26). Thus, changes in [Ca²⁺], due to Na⁺/Ca²⁺ exchange are clearly detectable when the intracellular Na⁺ concentration is elevated, supporting the hypothesis that Na⁺/Ca²⁺ exchange contributes to Ca²⁺ homeostasis in endothelial cells.

The present study was designed to elucidate the functional relevance of Na⁺/Ca²⁺ exchange for Ca²⁺-dependent activation of eNOS in cultured porcine aortic endothelial cells. We provide evidence for facilitation of eNOS activation due to reversed mode Na⁺/Ca²⁺ exchange.

**EXPERIMENTAL PROCEDURES**

**Materials**—FURA-2/AM was obtained from Lambda Fluorescence Technology (Graz, Austria). L-[^3]H Arginine hydrochloride (57 Ci/mmol) and the ECL Western blotting detection system were from Amersham Pharmacia Biotech. SK&F 96365 (1-[1-(2,4,5-[^3]H) arginine] (200 al FURA-2/AM) was purchased from Lambda Fluorescence Technology, Puchheim, Austria, and 3,4-dichlorobenzamil hydrochloride (DCB) was from Molecular Probes, Eugene, Oregon. Monoclonal anti-caveolin-1 antibody was from Transduction Laboratories purchased through Margaritella (Vienna, Austria), and polyclonal anti-NCX antibody was from Swant (Bellinzona, Switzerland). Preliminary experiments were performed with a monoclonal anti-NCX antibody kindly provided by H. Porzig (Bern, Switzerland). Polyclonal anti-eNOS antibody was raised in rabbits against purified bovine eNOS (27). All other compounds including secondary antibodies were purchased from Sigma.

**Cell Culture**—Porcine aortic endothelial cells were isolated as described (28) and cultured at 37 °C, 5% CO₂ up to 3 passages in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum and 3-imidazole hydrochloride was dissolved in 0.1 ml of 1N NaOH for the determination of total protein or in 6% sucrose and the homogenate was adjusted to 45% sucrose by adding 2 ml of 90% Laemmli buffer (30-32) at 11.2 m[M] [Ca²⁺]free incubation buffer evoked a transient rise in intracellular free Ca²⁺ that was maintained at a steady level for 3 min, incubation was terminated, and conversion of incorporated L-[[^3]H]arginine into L-[[^3]H]citrulline was determined as described above.

**Immunofluorescence Microscopy**—Endothelial cells, cultured on glass coverslips, were washed twice with PBS and fixed in PBS containing 3.7% formaldehyde and 1.5% methanol for 10 min at room temperature (35). After two washes with 0.2% Triton X-100 in PBS, cells were preincubated at 37 °C in PBS containing 0.3% Triton X-100, 1% bovine serum albumin, and 1% goat serum (blocking buffer). After 45 min, the antibody against eNOS (final dilution 1/10) or NCX (final dilution 1/500) was added, and cells were incubated for 90 min at 37 °C. Cells were then washed three times with blocking buffer and incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG in blocking buffer (dilution 1/200) for 30 min at 37 °C. After 2 washes with 0.2% Triton X-100 in PBS and 1 wash with PBS, cells were mounted in PBS containing 50% glycerol and 0.1% p-phenylenediamine. Images were obtained with a Leica TCS 4D confocal microscope equipped with an Ar/Kr laser and set up with the appropriate filter set for fluorescein isothiocyanate detection (488 nm excitation, TK515 beam splitter, BP-fluorescein isothiocyanate emission filter).

**RESULTS**

Fig. 1 shows a representative experiment in which endothelial Ca²⁺ stores were depleted with thapsigargin, followed by induction of capacitative Ca²⁺ entry by CaCl₂. The addition of 0.1 μM thapsigargin to endothelial cells suspended in nominally Ca²⁺-free incubation buffer evoked a transient rise in intracellular free Ca²⁺ levels ([Ca²⁺]i) due to the release of Ca²⁺ from intracellular stores. After [Ca²⁺]i had declined back to basal levels, capacitative Ca²⁺ entry was induced by adding increasing concentrations of CaCl₂, resulting in a stepwise increase in [Ca²⁺]i. To determine the correlation between extracellular free Ca²⁺ ([Ca²⁺]o) and the extracellular Ca²⁺ concentrations of CaCl₂ ([Ca²⁺]i), we measured Ca²⁺-sensitive electrode in parallel experiments, and [Ca²⁺]i was plotted against the respective steady-state levels of [Ca²⁺]o (Fig. 2A). The data showed that [Ca²⁺]i increased from a basal level of 162 ± 12 nm (mean ± S.E., n = 12) at 20 mM CaCl₂, up to 663 ± 23 nm (mean ± S.E., n = 12) at 11.2 mM CaCl₂, under control conditions.
To test for a contribution of NCX in Ca\(^{2+}\) homeostasis, endothelial cells were loaded with Na\(^{+}\) by incubation with the Na\(^{+}\) ionophore monensin, which is known to promote Ca\(^{2+}\) entry via reversed mode Na\(^{+}\)/Ca\(^{2+}\) exchange (26, 36). Alternatively, Na\(^{+}\) was omitted from the extracellular solution and replaced by choline chloride to avoid Na\(^{+}\) loading of the cells. As shown in Fig. 2A, replacement of Na\(^{+}\) by choline chloride in the extracellular solution did not change the correlation between the extracellular and the corresponding intracellular Ca\(^{2+}\) levels under control conditions. Preincubation of endothelial cells with the Na\(^{+}\)-ionophore monensin (1 \(\mu\)M) in Na\(^{-}\)-containing solution markedly reduced [Ca\(^{2+}\)]\(_{i}\), from 663 \(\pm\) 23 nM to 278 \(\pm\) 27 nM (mean \(\pm\) S.E., \(n = 8\)–12) at 11.2 mM [Ca\(^{2+}\)]\(_{i}\), indicating that Na\(^{+}\) loading inhibits rather than augments Ca\(^{2+}\) entry. Thus, an involvement of reversed mode Na\(^{+}/Ca^{2+}\) exchange in homeostasis of overall [Ca\(^{2+}\)]\(_{i}\), was not detectable in these experiments. Since a monensin-induced reduction of [Ca\(^{2+}\)]\(_{i}\), was not observed in Na\(^{-}\)-free buffer (Fig. 2A), the effect of monensin was presumably due to Na\(^{+}\) entry and consequent membrane depolarization, which is known to suppress store-operated Ca\(^{2+}\) entry.

The addition of CaCl\(_{2}\) to store-depleted endothelial cells increased eNOS activity as expected from Ca\(^{2+}\) entry (cf. Fig. 2A). In the presence of monensin, the discrepancy between the effects of extracellular Ca\(^{2+}\) on [Ca\(^{2+}\)]\(_{i}\), and eNOS activity was even more pronounced. As shown in Fig. 2B (open squares), Na\(^{+}\) loading with the Na\(^{+}\) ionophore potentiated the effect of extracellular Ca\(^{2+}\) on eNOS activity despite a marked reduction in overall [Ca\(^{2+}\)]\(_{i}\), (cf. Fig. 2A). Similar to the monensin-induced reduction of [Ca\(^{2+}\)]\(_{i}\), the effect of the Na\(^{+}\) ionophore on eNOS activity was completely abolished when extracellular Na\(^{+}\) was replaced by choline chloride (Fig. 2B, filled circles). To evaluate the Ca\(^{2+}\) dependence of eNOS activation under the various conditions, a correlation was drawn between L-citrulline formation and [Ca\(^{2+}\)]\(_{i}\), (Fig. 2C). The data revealed that, under control conditions, half-maximal activation of eNOS occurred at a [Ca\(^{2+}\)], of \(-310\) nM, whereas the EC\(_{50}\), was \(-390\) nM in nominal Na\(^{-}\)-free buffer. Pretreatment of the cells with monensin in the presence of Na\(^{+}\) resulted in a pronounced leftward shift of the concentration response curve (EC\(_{50}\), \(-210\) nM), but the Na\(^{+}\)-ionophore had no effect in the absence of extracellular Na\(^{+}\) (EC\(_{50}\), \(-390\) nM).

To test whether the monensin-induced facilitation of eNOS activation was due to a change in cellular Na\(^{+}\) gradients or rather a general phenomenon associated with membrane depolarization and/or inhibition of Ca\(^{2+}\) entry, we investigated the effects of K\(^{+}\)-induced membrane depolarization and inhibition of Ca\(^{2+}\) entry by SK&F 96365. As evident from Fig. 3A, treatment of the cells with 30 \(\mu\)M SK&F 96365 markedly reduced Ca\(^{2+}\) entry ([Ca\(^{2+}\)]\(_{i}\), \(-294\) \(\pm\) 14 nM (mean \(\pm\) S.E., \(n = 6\)) at 11.2 mM [Ca\(^{2+}\)]\(_{i}\)). A similar, albeit less pronounced effect was observed when experiments were performed in high K\(^{+}\) buffer ([Ca\(^{2+}\)]\(_{i}\), \(-531\) \(\pm\) 28 nM (mean \(\pm\) S.E., \(n = 6\)) at 10.3 mM [Ca\(^{2+}\)]\(_{i}\)). In accordance with their effects on [Ca\(^{2+}\)]\(_{i}\), both SK&F 96365 and KCl diminished the stimulation of eNOS by extracellular Ca\(^{2+}\) (Fig. 3B). Consequently, the correlation between L-citrulline formation and [Ca\(^{2+}\)]\(_{i}\), was neither affected by SK&F 96365 nor by KCl (Fig. 3C), demonstrating that the potentiation of Ca\(^{2+}\)-dependent eNOS activation observed in Na\(^{-}\)-loaded endothelial cells was not related to diminished Ca\(^{2+}\) entry and/or membrane depolarization.

Thus, Na\(^{+}\)-loading-induced facilitation of endothelial NO synthesis appears to be mediated either by a direct effect of Na\(^{+}\) ions on the enzyme or by a local elevation of free [Ca\(^{2+}\)], due to reversed mode Na\(^{+}\)/Ca\(^{2+}\) exchange, which is not detectable by measurement of overall Ca\(^{2+}\) signals. Direct effects of
Na⁺ on the Ca²⁺ sensitivity of eNOS were studied by measuring Ca²⁺-dependent activation of the enzyme in homogenates of endothelial cells. The data revealed that Na⁺ ions up to 100 mM failed to modify the effects of Ca²⁺ on enzyme activity (EC₅₀ for Ca²⁺ = 0.19 ± 0.02 μM and 0.20 ± 0.03 μM (mean ± S.E., n = 4 each) in the absence and presence of 100 mM Na⁺, respectively). We, therefore, tested whether DCB, an inhibitor of Na⁺/Ca²⁺ exchange, influences the effects of Na⁺ loading. For comparison, amiloride, a structural analog of DCB that barely affects Na⁺/Ca²⁺ exchange but inhibits Na⁺/H⁺ exchange and nonselective cation channels (37), was studied. Since both compounds interfered with the fluorometric detection of [Ca²⁺], the respective experiments were confined to the measurements of L-citrulline formation. As shown in Fig. 4, treatment of endothelial cells with 30 μM amiloride did not modify the effects of extracellular Ca²⁺ on eNOS activity in Na⁺-loaded cells (EC₅₀ ~ 0.3 mM). However, in the presence of 30 μM DCB, the Ca²⁺ sensitivity of l-citrulline formation was markedly reduced, resulting in an EC₅₀ of ~1.5 mM, which is comparable to the value observed in Na⁺-free buffer. These data strongly support the concept that Na⁺-dependent facilita-

FIG. 3. Effects of K⁺ and SK&F 96365 on overall [Ca²⁺]i, and eNOS activation. Endothelial cells were preincubated with 0.1 mM thapsigargin in nominal Ca²⁺-free buffer containing 100 mM Na⁺ (open circles), 100 mM Na⁺ plus 30 μM SK&F 96365 (filled circles) or 20 mM Na⁺ plus 100 mM K⁺ (open squares). After 15 min, CaCl₂ was added (final concentrations ~0.01 to ~10 mM), and overall [Ca²⁺]i, or eNOS activity was monitored as described under “Experimental Procedures.” The extracellular concentrations of Ca²⁺ were measured with a Ca²⁺-sensitive electrode in parallel experiments and plotted against the respective steady-state levels of [Ca²⁺], (panel A) or the formation of L-citrulline (panel B). In panel C, the correlation between l-citrulline formation and [Ca²⁺], is shown. Data are mean values ± S.E. of 6 to 12 experiments.

FIG. 4. Effects of amiloride and DCB on eNOS activation in Na⁺-loaded cells. Endothelial cells were preincubated with 0.1 mM thapsigargin and 1 μM monensin in nominal Ca²⁺-free buffer containing 100 mM Na⁺ (open circles), 100 mM Na⁺ plus 30 μM amiloride (filled circles), 100 mM Na⁺ plus 30 μM DCB (open squares), or 100 mM choline chloride (filled squares). After 15 min, CaCl₂ was added (final concentrations ~0.01 to ~10 mM), and eNOS activity was monitored as described under “Experimental Procedures.” The extracellular concentrations of Ca²⁺ were measured with a Ca²⁺-sensitive electrode in parallel experiments and plotted against the respective l-citrulline data. Data are mean values ± S.E. of 6 experiments.
port our concept that Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange may play a prominent role in the regulation of eNOS activity.

**DISCUSSION**

As several previous reports have indicated the contribution of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange to Ca\textsuperscript{2+} homeostasis in endothelial cells (11, 26, 36), it was of interest to study the role of NCX in cellular regulation of eNOS activity. Thereby, overall intracellular Ca\textsuperscript{2+} levels and L-citrulline formation were determined in parallel. The readdition of extracellular Ca\textsuperscript{2+} after store depletion with thapsigargin in nominally Ca\textsuperscript{2+}-free incubation buffer allowed for reproducible and sustained elevation of \([\text{Ca}^{2+}]_i\), and for reliable determination of Ca\textsuperscript{2+} concentration response curves. Two approaches were used to test for a contribution of NCX in the control of eNOS: (i) the cellular Na\textsuperscript{+} gradient was altered by use of monensin, and (ii) NCX was selectively blocked by DCB.

**Na\textsuperscript{+} Loading Facilitates Ca\textsuperscript{2+}-dependent Activation of eNOS**—Na\textsuperscript{+} loading of endothelial cells is an intervention that was used previously to demonstrate NCX-mediated Ca\textsuperscript{2+} signals in endothelial cells (26, 36). In those studies, transient rises in overall \([\text{Ca}^{2+}]_i\) were observed when extracellular Na\textsuperscript{+} was removed after agonist stimulation of Na\textsuperscript{+}-loaded endothelial cells. However, we observed that Na\textsuperscript{+} loading reduced overall \([\text{Ca}^{2+}]_i\) of thapsigargin-stimulated cells. This result is probably explained by the fact that in the present study total steady-state levels of \([\text{Ca}^{2+}]_i\) were analyzed instead of the increments of \([\text{Ca}^{2+}]_i\) induced by Na\textsuperscript{+} removal. The total, steady-state level of \([\text{Ca}^{2+}]_i\) after stimulation with thapsigargin is the result of Ca\textsuperscript{2+} entry via store-operated Ca\textsuperscript{2+} channels and concomitant Ca\textsuperscript{2+} transport via exchange mechanisms (NCX, Ca\textsuperscript{2+}-ATPase). Na\textsuperscript{+} loading promotes Ca\textsuperscript{2+} entry via reversed mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (26, 36) but suppresses store-operated Ca\textsuperscript{2+} entry via a reduction of the electrochemical gradient (25, 39). In our experimental setup, inhibition of store-operated Ca\textsuperscript{2+} entry was apparently the prominent effect of Na\textsuperscript{+} loading and masked the contribution of other Ca\textsuperscript{2+} transport systems, e.g. Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Treatment of endothelial cells with monensin augmented NOS activity without detectable changes of \([\text{Ca}^{2+}]_i\), resulting in a striking change in the correlation between overall \([\text{Ca}^{2+}]_i\), and eNOS activity. This
facilitation of eNOS stimulation was clearly dependent on the entry of Na\(^+\) ions, since preincubation of endothelial cells with monensin in the absence of Na\(^+\) failed to affect Ca\(^{2+}\)-dependent enzyme activation. Thus, our results provide the first evidence for a role of Na\(^+\) ions in cellular regulation of eNOS.

Mechanism of Na\(^+\)-dependent Facilitation of eNOS Activation—Various mechanisms such as direct modulation of eNOS activity by Na\(^+\) ions, membrane depolarization, changes in intracellular pH, or Na\(^+\)/Ca\(^{2+}\) exchange may explain the observed Na\(^+\)-dependent facilitation of eNOS activation. A direct modulatory effect of Na\(^+\) ions on Ca\(^{2+}\) sensitivity of the enzyme was excluded in experiments with cell-free eNOS preparations. Moreover, the Na\(^+\) entry-induced membrane depolarization itself is apparently not involved in promotion of eNOS activation, since the effect of monensin was not mimicked by elevation of extracellular K\(^+\). Similarly, a mechanism based on changes in intracellular pH can be excluded, since Na\(^+\) loading did not significantly affect intracellular pH of endothelial cells. Moreover, amiloride, a potent inhibitor of Na\(^+\)/H\(^+\) exchange, did not prevent the effects of monensin. In contrast to amiloride, the structural analog DCB, which is a potent inhibitor of Na\(^+\)/Ca\(^{2+}\) exchange, completely prevented Na\(^+\)-induced facilitation of eNOS activation. These results strongly support the view that the observed effects of Na\(^+\) loading were indeed due to reversed mode Na\(^+\)/Ca\(^{2+}\) exchange. Since we did not detect an elevation of overall [Ca\(^{2+}\)], in Na\(^+\)-loaded cells, the increase in [Ca\(^{2+}\)], induced by Na\(^+\)/Ca\(^{2+}\) exchange may be restricted to the subplasmalemmal region. It appears reasonable to speculate about a buildup of a subcellular Ca\(^{2+}\) gradient, since such preferential elevation of subplasmalemmal [Ca\(^{2+}\)] has been also observed upon activation of endothelial cells with bradykinin or sodium fluoride.

Presence of Endothelial NCX in Caveolae—The hypothesis that Na\(^+\) loading enhances eNOS activity via reversed mode Na\(^+\)/Ca\(^{2+}\) exchange and focal rises in subplasmalemmal [Ca\(^{2+}\)], requires the assumption that the involved NCX protein is located in close proximity of eNOS. Since regulation of eNOS is likely to take place in caveolae, it appeared of interest to test for the presence of NCX in these specialized plasma membrane domains. Separation of endothelial cell homogenates on a discontinuous sucrose gradient revealed the presence of both eNOS and NCX in the caveolin-1-positive fraction. In accordance with a previous report, eNOS was also found in higher density fractions, presumably representing Golgi-associated and cytosolic enzyme (38). Interestingly, the proportion of caveolae-associated eNOS reported by these authors was ~35% and, thus, considerably lower than observed in the present study (~90%). This variation in the subcellular distribution may be explained by variability of eNOS localization due to species differences, type of endothelial cells, or culture conditions (1, 40).

The predominant localization of eNOS in caveolae observed in our study was confirmed by immunofluorescence microscopy. Consistent with previous immunofluorescence staining of eNOS in bovine aortic endothelial cells (40), we observed that the majority of cells exhibited a substantial immunoreactivity in the plasma membrane region, whereas a perinuclear staining was only found in a minority of cells. In contrast to this heterogeneous pattern of eNOS staining, the immunoreactivity against the NCX antibody did not exhibit considerable cell-to-cell variations; as in all cells investigated, a substantial staining in the regions of the plasma membrane, the Golgi, and the cytoskeleton was observed. Consistent with these immunofluorescence data, NCX was enriched in the plasma membrane and caveolae fractions of the sucrose gradient but was also present in higher density fractions. Albeit eNOS and NCX exhibit a clearly different pattern of subcellular localization, our results strongly suggest the presence of both proteins in caveolae. Since eNOS has been shown to interact with a variety of proteins present in caveolae (e.g. the structural protein caveolin-1 (5–7), the bradykinin receptor B2 (41), or the L-arginine transporter CAT-1 (42)), it is reasonable to speculate that eNOS may also form a complex with NCX. However, we did not observe a substantial coimmunoprecipitation of eNOS and NCX from endothelial cell lysates, indicating that both proteins apparently do not form a tight and stable complex.

Regulation of Endothelial Ca\(^{2+}\) Homeostasis by Na\(^+\) Ions—Based on our data, we suggest that Ca\(^{2+}\) concentrations at the cytoplasmic side of caveolae and, thus, eNOS activity is for a large part controlled by Na\(^+\)/Ca\(^{2+}\) exchange and propose the following model (Fig. 8). Ca\(^{2+}\)-dependent stimulation of eNOS in endothelial cells involves two distinct Ca\(^{2+}\) transport systems in the plasma membrane. Ca\(^{2+}\) channels, activated by depletion of intracellular Ca\(^{2+}\) stores, provide a general pathway for Ca\(^{2+}\) entry into activated endothelial cells. Albeit store-operated Ca\(^{2+}\) entry is the major determinant of the overall Ca\(^{2+}\) signal observed in agonist-stimulated endothelial cells, essential subcellular Ca\(^{2+}\) gradients are established by Na\(^+\)/Ca\(^{2+}\) exchange. An NCX protein is present in caveolae and, thus, in close proximity of eNOS. Thereby, NCX essentially contributes to local Ca\(^{2+}\) homeostasis and cellular control of eNOS activity. This interaction between eNOS and NCX allows for modulation of eNOS activity via the cellular Na\(^+\) gradient and, thus, via a variety of Na\(^+\) transport systems.

Physiological and Pathophysiological Implications—In the present study, Ca\(^{2+}\)-dependent activation of eNOS was primarily investigated in store-depleted endothelial cells. This method is widely accepted as a physiological relevant model of endothelial cell activation, since direct depletion of intracellular Ca\(^{2+}\) stores by inhibitors of endoplasmic Ca\(^{2+}\) -ATPase such as thapsigargin mimics the stimulation of phospholipase C-coupled receptors in terms of Ca\(^{2+}\)/NO signaling due to activation of the same Ca\(^{2+}\) entry pathway (8, 30). It is therefore conceivable to conclude that the effects of Na\(^+\) loading observed in thapsigargin-treated endothelial cells will take place similarly in cells activated by stimulation of phospholipase C-coupled receptors. Consistently, we observed that Na\(^+\) loading of endothelial cells also potentiates eNOS activation by bradykinin.

2 K. Groschner, unpublished observations.

3 M. Teubl and K. Schmidt, unpublished observations.
due to Ca\textsuperscript{2+} entry via reversed mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange.

In the absence of Na\textsuperscript{+} loading, forward mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange is likely to contribute to Ca\textsuperscript{2+} extrusion during cell activation (11). It appears reasonable to expect that even a moderate elevation of intracellular Na\textsuperscript{+}, in particular in association with membrane depolarization, may substantially suppress Ca\textsuperscript{2+} extrusion and, thus, facilitates Ca\textsuperscript{2+}-dependent activation of eNOS. The contribution of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange is likely to vary, depending on the ability of a physiological stimulus to induce Na\textsuperscript{+} loading (36). The recent demonstration of an inositol 1,4,5-trisphosphate-dependent, endothelial Na\textsuperscript{+} activation (11). It appears reasonable to expect that even a 

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