Na\textsuperscript{+}-K\textsuperscript{+} pump activation inhibits endothelium-dependent relaxation by activating the forward mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in mouse aorta

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The Na\textsuperscript{+}-K\textsuperscript{+} pump activation inhibits endothelium-dependent relaxation by activating the forward mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in mouse aorta. Am J Physiol Heart Circ Physiol 289: H2020–H2029, 2005. First published July 1, 2005; doi:10.1152/ajpheart.00908.2004.—The effect of Na\textsuperscript{+}-K\textsuperscript{+} pump activation on endothelium-dependent relaxation (EDR) and on intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) was examined in mouse aorta and mouse aortic endothelial cells (MAECs). The Na\textsuperscript{+}-K\textsuperscript{+} pump was activated by increasing extracellular K\textsuperscript{+} concentration ([K\textsuperscript{+}]\textsubscript{o}) from 6 to 12 mM. In aortic rings, the Na\textsuperscript{+} ionophore monensin evoked EDR, and this EDR was inhibited by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX; reverse mode) inhibitor KB-R7943. Monensin-induced Na\textsuperscript{+} loading or extracellular Na\textsuperscript{+} depletion (Na\textsuperscript{+} replaced by Li\textsuperscript{+}) increased [Ca\textsuperscript{2+}]\textsubscript{i} in MAECs, and this increase was inhibited by KB-R7943. Na\textsuperscript{+}-K\textsuperscript{+} pump activation inhibited EDR and [Ca\textsuperscript{2+}]\textsubscript{i} increase ([K\textsuperscript{+}]\textsubscript{o}), by 10.2 ± 2.2 % at 10 μM but did not exceed 30 μM. In current-clamped MAECs, an increase in [K\textsuperscript{+}]\textsubscript{o} from 6 to 12 mM depolarized the membrane potential, which was inhibited by ouabain, Ni\textsuperscript{2+}, or KB-R7943. In aortic rings, the concentration of cGMP was significantly increased by acetylcholine and decreased on increasing [K\textsuperscript{+}]\textsubscript{o} from 6 to 12 mM. This decrease in cGMP was significantly inhibited by pretreating with ouabain (100 μM), Ni\textsuperscript{2+} (300 μM), or KB-R7943 (30 μM). These results suggest that activation of the forward mode of NCX after Na\textsuperscript{+}-K\textsuperscript{+} pump activation inhibits Ca\textsuperscript{2+} mobilization in endothelial cells, thereby modulating vasomotor tone.

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First-strand cDNA was generated from total RNA using BcaBEST polymerase (Takara Shuzo). The specific oligonucleotide primers used for the PCR were 5'-CTTCTTCTTTCCCCATC-CTGTT-3' (sense) and 5'-GACTCTGACATTGCTAAGTTGC-3' (antisense). The size of the expected fragments was 783 bp. PCR was performed using Bca-optimized Taq polymerase (Takara Shuzo), and the conditionings used were as follows: initial denaturation for 1 min at 94°C, 35 amplification cycles (1 min at 94°C, 1.5 min at 55°C, and 1.5 min at 72°C for each cycle), a final extension of 10 min at 72°C, and rapid cooling to 4°C. PCR products were visualized on a 1% agarose gel using a 100-bp DNA ladder marker (New England BioLabs; Beverly, MA) as a standard.

Determination of mouse aorta cGMP levels. Mouse aortic rings, prepared as described above, were allowed to equilibrate in a chamber of a myograph, and one ring was mounted to record contractile response in the same chamber. The chamber was perfused at a flow rate of 2.5 ml/min with oxygenated (95% O₂-5% CO₂) Krebs-Ringer bicarbonate solution by using a peristaltic pump. Rings were precontracted with NE, and EDR was induced by 3 μM ACh. When the mounted ring showed an expected response, the other rings were frozen in liquid nitrogen, and cGMP levels were measured using cGMP assay kits (EIA kit, R&D Systems) following the manufacture's protocol. Briefly, frozen tissue samples in liquid nitrogen were ground to a fine powder using a stainless steel mortar. After the liquid

Fig. 1. Activation of the reverse mode of Na+/Ca2+ exchanger (NCX) by monensin-induced Na+ loading and extracellular Na+ depletion (Na+ replaced by Li+). A–D: monensin (M) relaxed endothelium-intact (E+; A, B, and D) and endothelium-denuded aortic rings (E–; C and D) in a concentration-dependent manner. The magnitude of the relaxation at each experiment was expressed as a percentage of the initial PGF2α-induced contraction. The magnitude of monensin-induced relaxation was compared with that of ACh (3 μM)-induced endothelium-dependent relaxation (EDR; D). In endothelium-intact rings, monensin-induced relaxations were 29.2 ± 4.7% (0.1 μM) and 58.6 ± 3.3% (1 μM) of ACh-induced relaxation, respectively. E–G: monensin-induced Na+ loading or extracellular Na+ depletion significantly increased [Ca2+], (E and F) and extracellular Na+ depletion augmented ATP-induced intracellular Ca2+ concentration ([Ca2+]i) increase in mouse aortic endothelial cells (MAECs). C, control; M, monensin. H: RT-PCR studies of MAECs for NCX1. M, 100-bp ladder size standard. **Statistical significance (P < 0.01).
RESULTS

Presence of NCX in MAECs. The Na⁺ ionophore monensin relaxed precontracted endothelium-intact aortic rings in a concentration-dependent manner (Fig. 1, A, B, and D). Monensin induced 25.2 ± 4.3% relaxation at 0.1 μM, and this relaxation was completely inhibited by KB-R7943 (Fig. 1A). In contrast, endothelium-denuded aortic rings (Fig. 1C) or endothelium-intact aortic rings pretreated with the nitric oxide (NO) synthase inhibitor l-NAME (30 μM) (data not shown) showed no relaxation on administration of monensin (0.1 μM), indicating that the KB-R7943-sensitive, monensin-induced relaxation was EDR. On the other hand, monensin relaxed not only precontracted endothelium-intact aortic rings but also endothelium-denuded aorta at high concentrations (>1 μM). Monensin (1 μM) induced 56.5 ± 4.8 and 10.3 ± 3.1% relaxation in endothelium-intact and -denuded aortic rings, respectively (Fig. 1, B–D), and this relaxation was significantly greater in endothelium-intact aortic rings (Fig. 1D). Monensin-induced relaxation of endothelium-denuded aortic rings was not inhibited by KB-R7943 (data not shown). We then examined the effects of monensin-induced Na⁺ loading and extracellular Na⁺ depletion (Na⁺ replaced by Li⁺) on [Ca²⁺] in MAEC (Fig. 1, E and F). With monensin application or extracellular Na⁺ depletion, [Ca²⁺], was increased from 0.15 ± 0.02 to 0.23 ± 0.03 μM or from 0.16 ± 0.02 to 0.24 ± 0.02 μM, respectively (n = 10), and these increases were inhibited by KB-R7943 (data not shown). In addition, extracellular Na⁺ depletion increased [Ca²⁺] in ATP-stimulated MAECs (Fig. 1G). Increased [Ca²⁺], by ATP application was further in-
creased by extracellular Na⁺ depletion, and this Na⁺ depletion-induced [Ca²⁺]ᵢ increase was inhibited by KB-R7943.

Inasmuch as the above results suggested the presence of NCX, we next performed RT-PCR using mRNA of freshly isolated MAECs to examine whether the cells contain a molecular candidate for NCX. As a complete sequence for mouse NCX has been published, we performed RT-PCR using primers based on this NCX sequence. Figure 1H shows that an abundant RT-PCR product was found at the expected molecular size using a PCR primer set, indicating that NCX1 mRNA is expressed in MAECs.

[Na⁺]-dependent mechanism? In a previous study (26), we reported that EDR and agonist-induced [Ca²⁺]ᵢ increase in various endothelial cells were inhibited by increasing extracellular concentrations of K⁺ or Cs⁺ within a millimolar range (K⁺-induced inhibition), and this K⁺-induced inhibition was inhibited by the Na⁺-K⁺ pump inhibitor ouabain. These data suggested that Na⁺-K⁺ pump activation evoked K⁺-induced inhibition. Because Na⁺-K⁺ pump activation reduces [Na⁺]ᵢ, a decrease in [Na⁺]ᵢ might be an initial step in K⁺-induced inhibition. Thus we tested the effect of monensin-induced Na⁺ loading on K⁺-induced inhibition.

Precontracted endothelium-intact aortic rings were relaxed by ACh application. ACh-induced EDR was abolished by increasing [K⁺]ₒ from 6 to 12 mM in a reversible manner (K⁺-induced inhibition of EDR) (Fig. 2A). This K⁺-induced inhibition of EDR was almost completely abolished by monensin (0.1 µM) pretreatment for 30 min (Fig. 2B). In addition, 1 µM monensin completely inhibited K⁺-induced inhibition of EDR (Fig. 2, C and D). We then examined the effect of monensin-induced Na⁺ loading on K⁺-induced inhibition of [Ca²⁺]ᵢ increase. ATP increased [Ca²⁺]ᵢ, and this increased

![Figure 3](http://ajpheart.physiology.org/)

Fig. 3. Inhibition of K⁺-induced inhibition by the NCX (forward and reverse mode) inhibitor Ni²⁺. A: Ni²⁺ inhibited K⁺-induced inhibition of EDR in a concentration-dependent manner. B: aortas used in A were pretreated with N⁶-nitro-L-arginine methyl ester (L-NAME). Note that Ni²⁺ did not relax the aortic ring. C: K⁺-induced inhibition in control rings. NE, norepinephrine. D and E: aortas used in C were pretreated with Ni²⁺. The magnitude of the relaxation at each experiment was expressed as a percentage of the initial NE-induced contraction, and K⁺-induced inhibition of EDR was significantly reduced by Ni²⁺ pretreatment (E). In addition, this pretreatment significantly potentiated EDR (E). F: Ni²⁺ also inhibited K⁺-induced inhibition of [Ca²⁺]ᵢ increase. Oxygenated (100% O₂) HEPES-buffered solution was used in Ni²⁺ experiments. *P < 0.05, ***P < 0.001.
[Ca\(^{2+}\)]_i reduced by increasing [K\(^+\)]_o from 6 to 12 mM in a reversible manner (K\(^+\)-induced inhibition of [Ca\(^{2+}\)]_i increase) (Fig. 2E). This K\(^+\)-induced inhibition of [Ca\(^{2+}\)]_i increase was abolished by pretreating with monensin (10 \(\mu\)M) for 5 min (Fig. 2F). These results suggest that intracellular Na\(^+\) depletion by Na\(^+\)-K\(^+\) pump activation evokes K\(^+\)-induced inhibition.

**Contribution of the forward mode of NCX to K\(^+\)-induced inhibition.** [Ca\(^{2+}\)]_i in endothelial cells is increased during EDR. Because low [Na\(^+\)]_i and high [Ca\(^{2+}\)]_i might activate the forward mode of NCX, we tested the effect of NCX inhibitors on K\(^+\)-induced inhibition. After evoking K\(^+\)-induced inhibition of EDR, the NCX (forward and reverse mode) inhibitor Ni\(^{2+}\) was applied. Rings, recontracted by extracellular K\(^+\), were slightly relaxed by 100 \(\mu\)M Ni\(^{2+}\) and markedly relaxed by 300 \(\mu\)M Ni\(^{2+}\) (Fig. 3A). In contrast, 300 \(\mu\)M Ni\(^{2+}\) did not relax endothelium-intact aortic rings pretreated with l-NAME (30 \(\mu\)M) (Fig. 3B) or endothelium-denuded aortic rings (data not shown). These results suggest that the NCX inhibitor Ni\(^{2+}\) inhibits K\(^+\)-induced inhibition of EDR. Pretreatment with Ni\(^{2+}\) for 5 min also inhibited K\(^+\)-induced inhibition of EDR (Fig. 3, C–E). In control rings, EDR was almost completely inhibited by increasing [K\(^+\)]_o from 6 to 12 mM. EDR of 90.9 \(\pm\) 4.1% was reduced to 7.7 \(\pm\) 3.2% by increasing [K\(^+\)]_o, \((n = 6)\). In contrast, when the aortic rings were pretreated with 100 \(\mu\)M Ni\(^{2+}\), EDR was only partially inhibited by increasing [K\(^+\)]_o from 6 to 12 mM. EDR of 94.6 \(\pm\) 5.1% was reduced to 60.2 \(\pm\) 4.7% by increasing [K\(^+\)]_o. The inhibitory effect of Ni\(^{2+}\) on K\(^+\)-induced inhibition was further augmented by increasing the Ni\(^{2+}\) concentration from 100 to 300 \(\mu\)M (Fig. 4).

**Fig. 4.** Inhibition of K\(^+\)-induced inhibition of EDR by the NCX (forward and reverse mode) inhibitor 2,4'-dichlorobenzamil (DCB). The magnitude of the relaxation at each experiment was expressed as a percentage of the initial NE-induced contraction. A: K\(^+\)-induced relaxation in control. B–D: aortas used in A were pretreated with 10 \(\mu\)M DCB (B) or 30 \(\mu\)M DCB (C). K\(^+\)-induced inhibition was significantly and concentration dependently reduced by these pretreatment (D). E–G: EDR induced by a low concentration of ACh (0.3 \(\mu\)M) in control rings (E) and after DCB pretreatment (F). The magnitude of EDR was significantly increased by pretreating with DCB (+DCB; G). **P < 0.01, ***P < 0.001.
3D). These results support the suggestion that the NCX inhibitor Ni\(^{2+}\) inhibits K\(^{+}\)-induced inhibition of EDR. We then examined the effect of Ni\(^{2+}\) on K\(^{+}\)-induced inhibition of \([\text{Ca}^{2+}]_{i}\) increase. Ni\(^{2+}\) restored the reduced \([\text{Ca}^{2+}]_{i}\) by extracellular K\(^{+}\) (Fig. 3F), which suggests that Ni\(^{2+}\) inhibits K\(^{+}\)-induced inhibition of \([\text{Ca}^{2+}]_{i}\) increase.

Like Ni\(^{2+}\), another NCX (forward and reverse mode) inhibitor, DCB, also inhibited K\(^{+}\)-induced inhibition of EDR (Fig. 4). In control rings, EDR was almost completely inhibited by extracellular K\(^{+}\) (Fig. 4A and D). EDR of 81.0 ± 1.6% was reduced to 3.1 ± 1.9% by increasing \([\text{K}^{+}]_{o}\) from 6 to 12 mM (n = 6). The aortic rings were then pretreated with DCB for 10 min. DCB pretreatment inhibited K\(^{+}\)-induced inhibition of EDR in a concentration-dependent manner. Pretreatment with 10 \(\mu\)M DCB partially inhibited K\(^{+}\)-induced inhibition of EDR. EDR of 88.8 ± 1.0% was reduced to 50.1 ± 5.1% by increasing \([\text{K}^{+}]_{o}\) from 6 to 12 mM (Fig. 4B and D). Furthermore, pretreatment with 30 \(\mu\)M DCB almost completely abolished K\(^{+}\)-induced inhibition of EDR (Fig. 4C). EDR of 89.0 ± 1.7% was slightly reduced to 81.0 ± 1.2% by increasing \([\text{K}^{+}]_{o}\) from 6 to 12 mM. In addition, pretreatment with Ni\(^{2+}\) or DCB potentiated EDR (Figs. 3E and 4D). EDRs of 90.9 ± 4.1% and 81.0 ± 1.6% were significantly increased to 94.6 ± 5.1% and 88.8 ± 1.0% by pretreating with Ni\(^{2+}\) and DCB, respectively. The potentiating effect of DCB on EDR was more evident, when a low concentration of ACh (0.3 \(\mu\)M) was used to induce EDR (Fig. 4E–G). The EDR of 34.6 ± 4.9% was significantly increased to 68.3 ± 4.1% by pretreating with DCB (n = 6).

KB-R7943 (the reverse mode inhibitor) did not inhibit K\(^{+}\)-induced inhibition of EDR or \([\text{Ca}^{2+}]_{i}\) increase at up to 10 \(\mu\)M (Fig. 5, B and E). When the aortic rings were pretreated with 10 \(\mu\)M KB-R7943, K\(^{+}\)-induced inhibition of EDR was still evoked (Fig. 5B). In contrast, KB-R7943 completely abolished K\(^{+}\)-induced inhibition of EDR at 30 \(\mu\)M (Fig. 5C). In addition, KB-R7943 inhibited K\(^{+}\)-induced inhibition of \([\text{Ca}^{2+}]_{i}\) increase.
Fig. 6. Effect of the Na\textsuperscript{+}-K\textsuperscript{+} pump inhibitor ouabain on the depolarized membrane potential by extracellular K\textsuperscript{+}. A and B: resting membrane potentials were depolarized by ouabain, when [K\textsubscript{o}] was maintained at 6 mM. C and D: effect of extracellular K\textsuperscript{+} on the membrane potential was inhibited by the Na\textsuperscript{+}-K\textsuperscript{+} inhibitor ouabain. The depolarized membrane potential by extracellular K\textsuperscript{+} was hyperpolarized by ouabain. **Statistical significance (P < 0.001).

Fig. 7. Effects of the NCX inhibitors Ni\textsuperscript{2+} and KB-R7943 on the depolarized membrane potential (V\textsubscript{M}) by extracellular K\textsuperscript{+}. A: resting membrane potentials were not changed by Ni\textsuperscript{2+} or KB-R7943, when [K\textsubscript{o}] was maintained at 6 mM. B and C: effect of extracellular K\textsuperscript{+} on the membrane potential was inhibited by the NCX inhibitor Ni\textsuperscript{2+}. The depolarized membrane potential was hyperpolarized by Ni\textsuperscript{2+}. D: KB-R7943 also hyperpolarized the depolarized membrane potential. **Statistical significance (P < 0.01).
at 30 μM (Fig. 5, E and F). A [Ca$^{2+}$]$_i$ of 0.12 ± 0.03 μM in the resting state was increased to 1.06 ± 0.19 μM by ATP, and this was reduced to 0.26 ± 0.12 μM by increasing [K+]o from 6 to 12 mM. The reduced [Ca$^{2+}$]$_i$ was increased to 0.63 ± 0.14 μM by 30 μM KB-R7943 (n = 8). These data indicate that Na$^{+}$-K$^{+}$ pump activation inhibits [Ca$^{2+}$]$_i$ increase in endothelial cells and EDR by activating the forward mode of NCX. On the other hand, KB-R7943 inhibited EDR and ATP-induced Ca$^{2+}$ transients in MAECs at high concentrations (>10 μM) (Fig. 5, A–D).

Concomitant activation of Na$^{+}$-K$^{+}$ pump and the forward mode of NCX. The above data suggests that the Na$^{+}$-K$^{+}$ pump and the forward mode of NCX are simultaneously activated by increasing [K+]o. Because activation of the Na$^{+}$-K$^{+}$ pump and the forward mode of NCX might affect the membrane potential, we examined the effect of ouabain and NCX blockers on the membrane potential. An increase in [K+]o from 6 to 12 mM depolarized the resting membrane potential from −29.8 ± 4.8 to −3.1 ± 3.8 mV in current-clamped MAECs (n = 14) (Figs. 6C and 7B), and this effect of extracellular K$^{+}$ was inhibited by ouabain (100 μM) or Ni$^{2+}$ (300 μM). Depolarized membrane potential was significantly hyperpolarized by ouabain or Ni$^{2+}$ from −2.1 ± 5.5 to −9.6 ± 5.1 mV (n = 12) (Fig. 6, C and D) and from −3.9 ± 4.1 to −15.4 ± 3.2 mV (n = 11) (Fig. 7, B and C), respectively. In addition, KB-R7943 also hyperpolarized this depolarized potential (Fig. 7D). In contrast, when [K+]o was maintained at 6 mM, the membrane potential was significantly depolarized by ouabain (100 μM) from −27.8 ± 5.8 to −20.6 ± 6.0 mV (n = 9) (Fig. 6, A and B) and not changed by Ni$^{2+}$ (300 μM, n = 8) or KB-R7943 (Fig. 7A). These data suggest that the Na$^{+}$-K$^{+}$ pump and the forward mode of NCX are simultaneously activated by extracellular K$^{+}$.

Effect of extracellular K$^{+}$ on cGMP levels. When [Ca$^{2+}$]$_i$ in endothelial cells is increased, endothelium-derived NO is released from endothelial cells. This released NO increases cGMP levels in vascular smooth muscle and thereby relaxes vascular smooth muscle. Thus we examined the effect of extracellular K$^{+}$ on cGMP levels in aortic rings (Fig. 8). The cGMP level in aortic rings exposed to NE (1 μM) was 1.37 ± 0.37 pmol/mg protein. When aortic rings were exposed to NE and then to ACh (3 μM), this level was significantly increased to 107.42 ± 11.11 pmol/mg protein. After K$^{+}$-induced inhibition of EDR was evoked, it fell significantly to 7.25 ± 1.36 pmol/mg protein (K$^{+}$-induced inhibition of cGMP production). K$^{+}$-induced inhibition of cGMP production was significantly inhibited by pretreating with ouabain (100 μM), Ni$^{2+}$ (300 μM), or KB-R7943 (30 μM).

**DISCUSSION**

This study provides new evidence concerning the roles of the Na$^{+}$-K$^{+}$ pump and the forward mode of NCX in endothelium-dependent vasorelaxation. In endothelial and vascular smooth muscle cells, an increase in [Ca$^{2+}$]$_i$, by various stimuli stimulates Ca$^{2+}$-activated K$^{+}$ channels (5, 22). This activation in turn induces an efflux of intracellular K$^{+}$ and may increase K$^{+}$ concentration transiently in the extracellular space close to the cell membrane. It was reported that endothelial cell stimulation by ACh raises the K$^{+}$ concentration in the myoendothelial space by 5.9 ± 1.0 mM (7). Therefore, when endothelial cells are stimulated, an increase in [K+]o might be enough to activate the Na$^{+}$-K$^{+}$ pump and the forward mode of NCX.

Subsequent activation of the forward mode of NCX after Na$^{+}$-K$^{+}$ pump activation is the mechanism of K$^{+}$-induced inhibition. In this study, we showed that Na$^{+}$-K$^{+}$ pump activation stimulates the forward mode of NCX and thereby inhibits intracellular Ca$^{2+}$ mobilization and endothelium-dependent vasorelaxation. Therefore, we suggest that subsequent activation of the forward mode of NCX after Na$^{+}$-K$^{+}$ pump activation is consistent with a previous finding that the NCX current reverses from an outward to an inward direction during Na$^{+}$-K$^{+}$ pump activation (3, 10). These findings revealed a dynamic interaction between the two Na$^{+}$ transport mechanisms, the Na$^{+}$-K$^{+}$ pump and NCX.

A [K+]o increase affects the membrane potential in various ways. A [K+]o increase might hyperpolarize membrane potential by activating the Na$^{+}$-K$^{+}$ pump. On the other hand, the membrane potential might be depolarized by activating the forward mode of NCX. Na$^{+}$-K$^{+}$ pump activation decreases [Na$^{+}$], and increases membrane potential through the Na$^{+}$-K$^{+}$ pump (10). Afterward, Na$^{+}$-K$^{+}$ pump activation reduces [Na$^{+}$], and increases membrane potential through the Na$^{+}$-K$^{+}$ pump (10). However, this study showed that Na$^{+}$-K$^{+}$ pump activation is consistent with a previous finding that the NCX current reverses from an outward to an inward direction during Na$^{+}$-K$^{+}$ pump activation (3, 10). These findings revealed a dynamic interaction between the two Na$^{+}$ transport mechanisms, the Na$^{+}$-K$^{+}$ pump and NCX.

**Fig. 8.** Effect of extracellular K$^{+}$ on cGMP concentration in mouse aortic smooth muscle. Increased cGMP concentrations were markedly reduced by increasing [K+]o. The inhibitory effect of extracellular K$^{+}$ on cGMP concentration was reduced by pretreating with ouabain, Ni$^{2+}$, or KB-R7943. **Statistical significance (P < 0.001).**


We found that the NCX inhibitors Ni\(^{2+}\) and DCB potentiated EDR. These results suggest that the forward mode of NCX operates to extrude Ca\(^{2+}\) from endothelial cells during EDR and thus reduces EDR. Therefore, EDR may be potentiated by inhibiting the forward mode of NCX. [Ca\(^{2+}\)]\(_i\), in endothelial cells is increased during EDR, and the increased intracellular Ca\(^{2+}\) stimulates Ca\(^{2+}\)-activated K\(^+\) channel in endothelial cells. In our previous study, we found that IK\(_{ca}\) current contributes to EDR (1). When endothelial cells are stimulated during EDR, K\(^+\) efflux through activated IK\(_{ca}\) may increase the K\(^+\) concentration in extracellular space close to the cell membrane within a millimolar range (7), and this increase in [K\(^+\)]\(_o\) may inhibit EDR by activating the Na\(^+\)-K\(^+\) pump and the forward mode of NCX.

K\(^+\)-induced inhibition of EDR was not blocked by a low concentration of KB-R7943 (up to 10 \(\mu\)M) but was blocked by a high concentration (30 \(\mu\)M). In addition, K\(^+\)-induced inhibition of [Ca\(^{2+}\)]\(_i\) increase was slightly inhibited by 10 \(\mu\)M KB-R7943 and markedly inhibited by 30 \(\mu\)M KB-R7943. These results suggest that 30 \(\mu\)M KB-R7943 is enough to abolish K\(^+\)-induced inhibition by inhibiting the forward mode of NCX1 completely in MAECs. This suggestion is consistent with a previous report that suggested that KB-R7943 inhibits the forward mode at high concentration (IC\(_{50}\) ≥ 30 \(\mu\)M) (13).

We found that KB-R7943 at low concentrations (up to 10 \(\mu\)M) did not change ATP-induced Ca\(^{2+}\) transients in MAECs, which is consistent with the finding that KB-R7943 at 1 \(\mu\)M did not inhibit EDR (25). In addition, we found that the subtype of NCX in MAECs is NCX1. These data suggest that the reverse mode of NCX does not contribute to EDR under physiological conditions, since KB-R7943 inhibits the reverse mode of NCX1 at low concentration (IC\(_{50}\) = 1.2–2.4 \(\mu\)M) (13). The reverse mode of NCX may contribute EDR in pathophysiological conditions such as Na\(^+\)-loaded conditions or hyponatremia (<100 mM) (25). On the other hand, KB-R7943 inhibited EDR at a high concentration (>10 \(\mu\)M). This inhibition can be explained by the finding that KB-R7943 (>10 \(\mu\)M) inhibited ATP-induced Ca\(^{2+}\) transients (Fig. 5D). It was reported that KB-R7943, at a high concentration (30 \(\mu\)M), affected other ion transporters such as Na\(^+\)/H\(^+\) exchanger, Ca\(^{2+}\) channels, Ca\(^{2+}\) pumps, Na\(^+\)-K\(^+\) pump, and Na\(^+\) channels (13, 23). Thus we suggest that KB-R7943 inhibits EDR by affecting these ion transporters on endothelial or smooth muscle cells at a high concentration.

**Physiological implications: role of NCX in EDR.** In this study, we found that the magnitude of EDR is significantly increased by the NCX (forward and reverse mode) inhibitors Ni\(^{2+}\) or DCB. This finding indicates that the forward mode of NCX reduces EDR in physiological conditions. In physiological conditions and in the absence of Na\(^+\) loading, NCX seems to contribute to Ca\(^{2+}\) extrusion during cell activation to prevent Ca\(^{2+}\) overload (11). Because K\(^+\) is released to the extracellular space through Ca\(^{2+}\)-activated K\(^+\) channels during endothelial cell activation, we speculate a dynamic interaction between Ca\(^{2+}\)-activated K\(^+\) channels, the Na\(^+\)-K\(^+\) pump, and NCX. During cell activation, the increased intracellular Ca\(^{2+}\) stimulates Ca\(^{2+}\)-activated K\(^+\) channel, which releases K\(^+\) to the extracellular space. This released K\(^+\) then activates Na\(^+\)-K\(^+\) pump and the forward mode of NCX subsequently, which prevents a further increase in [Ca\(^{2+}\)]. Through this mechanism, endothelial cells may inhibit an excessive increase in [Ca\(^{2+}\)], and sensitively control NO release. This control in NO release may be important, because NO is converted to toxic species such as peroxynitrite on reacting with oxygen radicals (4, 12, 15, 20, 21). This mechanism may enable endothelial cells to release an appropriate amount of NO by regulating increases in [Ca\(^{2+}\)]. On the other hand, as shown in this study, the reverse mode of NCX increases [Ca\(^{2+}\)] and evokes EDR in Na\(^+\)-loaded conditions (6, 25, 28). In pathophysiological situations such as under oxidative stress, excessive Na\(^+\) entry via redox-activated cation channels has been observed (2, 8). The resulting profound Na\(^+\) loading is expected to activate the reverse mode of NCX. Therefore, we suggest Na\(^+\)-Ca\(^{2+}\) exchange is a physiological and pathophysiological link between Na\(^+\) homeostasis and Ca\(^{2+}\)-associated cell signaling.

In conclusion, we show here that activation of the forward mode of NCX following Na\(^+\)-K\(^+\) pump activation decreases [Ca\(^{2+}\)] within endothelial cells and thus inhibits EDR. We suggest a dynamic interaction between Ca\(^{2+}\)-activated K\(^+\) channels, the Na\(^+\)-K\(^+\) pump, and NCX, which enables endothelial cells to sensitively control Ca\(^{2+}\) influx, and therefore [Ca\(^{2+}\)], and may be a mechanism by which endothelial cells sensitively modulate vasomotor activity.

**GRANTS**

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