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Sodium–calcium exchanger contributes to membrane hyperpolarization of intact endothelial cells from rat aorta during acetylcholine stimulation

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1 The role of sodium–calcium exchanger in acetylcholine (Ach)-induced hyperpolarization of intact endothelial cells was studied in excised rat aorta. The membrane potential was recorded using perforated patch-clamp technique.

2 The mean resting potential of endothelial cells was -44.1 ± 1.4 mV. A selective inhibitor of sodium–calcium exchanger benzamil (100 μM) had no significant effect on resting membrane potential, but reversibly decreased the amplitude of sustained Ach-induced endothelial hyperpolarization from 20.9 ± 1.4 to 5.7 ± 1.1 mV when applied during the plateau phase.

3 The blocker of reversed mode of the exchanger KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]-isothiourea methanesulfonate, 20 μM) reversibly decreased the amplitude of sustained Ach-induced hyperpolarization from 20.5 ± 2.9 to 7.5 ± 1.8 mV.

4 Introduction of tetraethylammonium (10 mM) in the continuous presence of Ach decreased the sustained phase of hyperpolarization from 17.9 ± 1.5 by 12.9 ± 0.9 mV. Subsequent addition of 20 μM KB-R7943 further depolarized endothelial cells by 4.8 ± 1.1 mV.

5 Substituting external sodium with *N*-methyl D-glucamine during the plateau phase of Ach-evoked hyperpolarization reversibly decreased the hyperpolarization from -61.8 ± 2.7 to -54.2 ± 1.9 mV. In the majority of preparations, the initial response to removal of external sodium was a transient further rise in the membrane potential of several mV. Sodium ionophore monensin hyperpolarized endothelium by 10.3 ± 0.7 mV.

6 The inhibitory effect of benzamil on Ach-induced endothelial sustained hyperpolarization was observed in endothelium mechanically isolated from smooth muscle.

7 These results suggest that the sodium–calcium exchanger of intact endothelial cells is able to operate in reverse following stimulation by Ach, contributing to sustained hyperpolarization. Myoendothelial electrical communications do not mediate the effect of blockers of sodium–calcium exchanger.

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Keywords: Endothelium; acetylcholine; membrane potential; sodium–calcium exchanger; gap junctions; benzamil; KB-R7943

Abbreviations: $[Ca^{2+}]_{sp}$, subplasmalemmal Ca^{2+} concentration; eNOS, endothelial nitric oxide synthase; 18α-GA, 18α-glycyrrhetinic acid; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate; K_{Ca}, calcium-activated potassium channels; NCX, sodium–calcium exchanger; NMDG, *N*-methyl D-glucamine; SK_{Ca}, small-conductance calcium-activated potassium channels

Introduction

Stimulation of endothelial cells by various vasoactive stimuli is known to elevate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The rise in $[Ca^{2+}]_i$ is a critical event in influencing numerous Ca^{2+}-dependent processes in vascular endothelium, including the synthesis and release of vasoactive substances. It has recently been hypothesized that stimulation of endothelial nitric oxide synthase (eNOS) that is located in specialized domains of the membrane, termed caveolae, may occur even without detectable rise in global $[Ca^{2+}]_i$ (Graier *et al*., 1998; Teubl *et al*., 1999) due to an increase in subplasmalemmal Ca^{2+} concentration ($[Ca^{2+}]_{sp}$). Endothelial $[Ca^{2+}]_{sp}$ is known to be in large part controlled by the sodium–calcium exchanger (NCX), which is localized in close proximity to eNOS (Teubl *et al*., 1999).

NCX of the plasma membrane is an electrogenic transporter that exchanges three Na^+ for every Ca^{2+}. Although the existence of NCX in endothelial cells has been demonstrated, there is some controversy regarding its properties in cultured and intact endothelial cells (Li & van Breemen, 1995) that has been attributed to altered membrane properties in cultured cells. Most existing knowledge about the NCX mechanism in endothelial cells comes from studies on cultured cells, which may behave differently from native endothelium. Indeed, there are substantial discrepancies in data regarding the physiological role of the exchanger in intact and cultured cells. In several studies, when cultured endothelial cells were used,

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substitution of external Na^+ had no impact on resting and bradykinin-induced increase in $[Ca^{2+}]_i$ (Schilling *et al*., 1988; Laskey *et al*., 1990), suggesting that NCX does not participate either in Ca^{2+} extrusion or entry under these conditions. Other reports demonstrated that inhibition of NCX increased stimulated Ca^{2+} entry (Klishin *et al*., 1998; Paltauf-Doburzynska *et al*., 1998; Domotor *et al*., 1999; Sedova & Blatter, 1999; Moccia *et al*., 2002), suggesting that NCX contributes to Ca^{2+} extrusion following stimulation of endothelial cells by agonists.

Data obtained from intact arteries, however, demonstrate that blockade of NCX by dichlorobenzamil decreases acetylcholine (Ach)-induced endothelium-dependent relaxation of rat aortic rings (Winquist *et al*., 1985; Schoeffter & Miller, 1986; Schneider *et al*., 2002), while endothelium-independent relaxation remains unaffected. These results suggest that NCX participates in Ca^{2+}-dependent release of NO following stimulation of endothelial cells by Ach. Discrepancies in these findings could either be attributed to restriction of changes in $[Ca^{2+}]_i$ within the subplasmalemmal area that are poorly detectable but critical for eNOS stimulation, to altered characteristics of the exchanger in cultured endothelial cells as compared with that of *in situ* endothelial cells, or to modulatory influence of smooth muscles on endothelium in intact vessels. Fluorescent properties of molecules of benzamil (Teubl *et al*., 1999; Moccia *et al*., 2002) restrict its usefulness in evaluation of the role of NCX in stimulated Ca^{2+} influx. However, electrogenicity of NCX could be indicative in this respect, since operation of this transporter results in a net movement of positive charge. Thus, the reversed mode of NCX is expected to contribute to the hyperpolarization of endothelial cells by two mechanisms: net efflux of positive charge per cycle and secondary stimulation of Ca^{2+}-dependent K^+ channels (K_{Ca}) due to an increase in $[Ca^{2+}]_{sp}$. These mechanisms should be able to alter the membrane potential of the cells during operation of NCX.

It is well established that the initial component of rise in $[Ca^{2+}]_i$ in endothelial cells following stimulation by Ach results from Ca^{2+} release from intracellular stores, whereas the delayed component, contributing to the plateau phase, depends on sustained extracellular Ca^{2+} influx across cell membrane. Elevation of $[Ca^{2+}]_i$ in endothelial cells evoked by Ach is responsible for membrane hyperpolarization due to stimulation of K_{Ca} (Busse *et al*., 1988; Marchenko & Sage, 1996; Wang & van Breemen, 1999). Similar to $[Ca^{2+}]_i$ increase, endothelial hyperpolarization in response to agonists is known to be biphasic, the initial hyperpolarization being attributable to release of stored Ca^{2+}, while the sustained hyperpolarization is attributable to Ca^{2+} entry. Endothelial K_{Ca} that underlie agonist-induced hyperpolarization of endothelial cells are therefore considered a reliable sensor of $[Ca^{2+}]_{sp}$ (Frieden *et al*., 2002). Moreover, endothelial hyperpolarization increases the electrochemical gradient for Ca^{2+}, thereby facilitating Ca^{2+} entry (Lückhoff & Busse, 1990).

The present study, therefore, was undertaken to investigate the sensitivity of Ach-induced delayed hyperpolarization of intact endothelial cells to the blockers of NCX. Here for the first time it is shown that blockade of NCX by using selective blockers, benzamil and KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)-phenyl]ethyl]isothiourea methanesulfonate), as well as by depletion of internal Na^+ inhibits the sustained hyperpolarization of *in situ* endothelial cells from rat aorta, suggesting that the reversed NCX contributes to membrane hyperpolarization and thus provides an additional Ca^{2+} entry pathway during stimulation by Ach.

Methods

Experiments were performed on 2–4 months old rats of both sexes. Rats were anesthetized by phorbol ester and killed by cervical dislocation. Thoracic aorta was excised and adherent fat removed. The thoracic aorta was placed in bubbled (95% O_2 and 5% CO_2) physiological saline solution of the following composition (mM): NaCl, 118.3; $NaHCO_3$, 25; KCl, 4.7; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.4; glucose, 11.1 (pH 7.4) and cut into ring segments of 2–3 mm in width. Before the experiment, the segment was cut open, and a strip was pinned to the rubber bottom of a 100 μl chamber and superfused by physiological saline solution at a rate of 0.5 ml min^{-1}. In Ca^{2+}-free extracellular solution, Ca^{2+} was substituted by 1 mM EGTA. The composition of HEPES-buffered solution was (in mM): NaCl, 145; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.4; glucose, 11.1. In Na^+-free medium, Na^+ was replaced by an equimolar amount of *N*-methyl-D-glucamine (NMDG).

The membrane potential was measured in intact aortic endothelial cells using a whole-cell configuration of a patch-clamp technique in a current clamp mode. The pipette viewed under a light microscope was lowered toward the luminal surface of the vessel strip, and its attachment with endothelium was recognized by a quick rise in the pipette resistance. Gigaohm contact was reached by suction. Electrical contact with the cytosol was established using nystatin. Nystatin was dissolved in DMSO: final nystatin concentration in the pipette solution was 200 μM. Patch pipettes, after being filled with the solution containing (mM) KCl 140, NaCl 10, EGTA 1, HEPES 10, had a resistance of 5–7 MΩ. pH of the pipette solution was adjusted to 7.2 by KOH. In a number of experiments, the membrane potential was measured from mechanically isolated endothelium as was described earlier (Marchenko & Sage, 1994b). Briefly, the aortal intima was separated from the media using a fine forceps and a needle and was re-fixed in the same chamber. The residue of the vessel was discarded. Experiments were conducted at room temperature (22–24°C). Pharmacological agents were applied to the preparation by bath perfusion. Quantitative data are expressed as means $\pm$ s.e. Statistical significance was evaluated by Student's *t*-test. *P*-values <0.005 were considered to be statistically significant.

The following chemicals were used: Ach chloride, phenylephrine hydrochloride, benzamil [(*N*-[benzylamidino]-3,5-diamino-6-chloropyrazine-carboxamide)], monensin, tetraethylammonium chloride, 18α-glycyrrhetinic acid (18α-GA) (all from Sigma). KB-R7943 was obtained from Tocris Cookson.

Results

Ca^{2+} dependence of Ach-induced endothelial hyperpolarization

The mean resting potential of endothelial cells from excised rat aorta in the present study was -44.1 ± 1.4 mV. Administration

of 2 μM Ach caused a maintained endothelial hyperpolarization to -64.8 ± 1.4 mV ($n = 32$) with a very slow decay in the continued presence of Ach (Figure 1a). No depolarization phase was observed in the present study. The membrane potential returned to baseline on washout of ACh.

To test the role of Ca^{2+} influx in the fluctuations of membrane potential, Ach-evoked responses were studied either in Ca^{2+}-free solution (no added Ca^{2+} and 1 mM EGTA) or in the presence of agonist-induced Ca^{2+} entry blocker, Ni^{+}. The superfusion of the vessel strip with Ca^{2+}-free solution caused a gradual depolarization of the endothelium by 13.2 ± 2.8 mV ($n = 5$). Subsequent Ach administration caused only a transient hyperpolarization with no plateau ($n = 5$) (Figure 1b). In the presence of 2 mM Ni^{2+}, which blocks different Ca^{2} entry pathways including NCX (Iwamoto & Shigekawa, 1988), Ach also evoked a short-lived hyperpolarization (Figure 1c). This is consistent with previous reports (Chen & Cheung, 1992; Wang & van Breemen, 1999) showing that sustained hyperpolarization is strictly dependent on Ca^{2+} entry from the extracellular space.

Effect of NCX blockers on sustained endothelial hyperpolarization

To evaluate the contribution of NCX to endothelial hyperpolarization induced by Ach, two distinct inhibitors of NCX, benzamil and KB-R7943, were employed. Benzamil administration (100 μM) had no significant effect on the resting membrane potential of endothelial cells ($n = 5$). However, in the presence of 100 μM benzamil, Ach (2 μM) induced only a short-lived hyperpolarization ($n = 3$) (Figure 2a). When benzamil was added during the plateau phase of hyperpolarization, its amplitude was markedly decreased. Thus, in this set of experiments, Ach hyperpolarized endothelial cells from the mean resting potential of -41.4 ± 1.4 mV to the mean plateau value of -62.3 ± 1.3 mV. Further benzamil administration in the continuous presence of Ach decreased the hyperpolarization to -47.1 ± 1.9 mV ($n = 15$) within 2 min (Figure 2b). In two cells, benzamil completely abolished the Ach-evoked hyperpolarization. The hyperpolarization was restored following removal of benzamil.

To test more specifically for the involvement of reversed mode of NCX in the sustained hyperpolarization in response to Ach, the drug KB-R7943 was employed. This recently developed agent preferentially blocks the reversed mode of NCX (Iwamoto *et al*., 1996; Watano *et al*., 1996). Similar to benzamil, KB-R7943 (20 μM) caused little changes in the resting membrane potential when applied within 2 min ($n = 5$), but markedly inhibited the delayed phase of hyperpolarization (Figure 3a). Thus, in this series of experiments, Ach (2 μM) hyperpolarized endothelial cells from -44.8 ± 2.3 to -65.3 ± 4.1 mV. Subsequent administration of 20 μM KB-R7943 reversibly inhibited the hyperpolarization to -52.3 ± 3.3 mV ($n = 4$) within 2 min throughout Ach application.

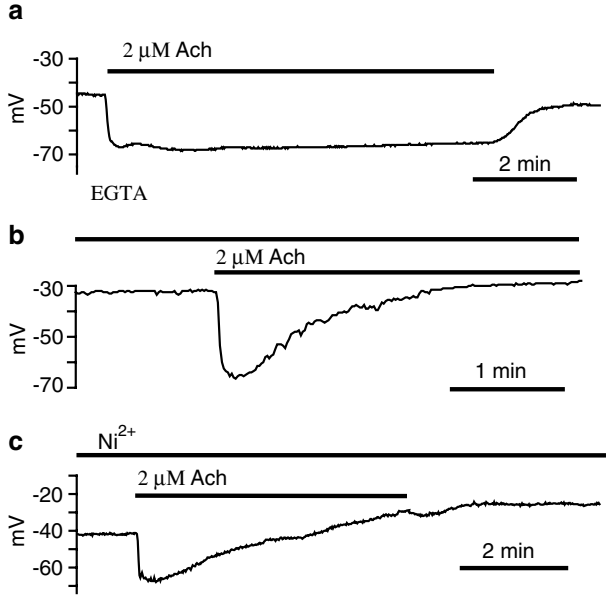

Figure 1 Sustained endothelial hyperpolarization in response to Ach requires extracellular Ca^{2+} entry. (a) A typical response of endothelial membrane potential to Ach in the presense of 2.4 mM extracellular Ca^{2+}. (b, c) Transient hyperpolarization in response to Ach (b) in Ca^{2+}-free solution and (c) in the presence of 2 mM Ni^{2+}.

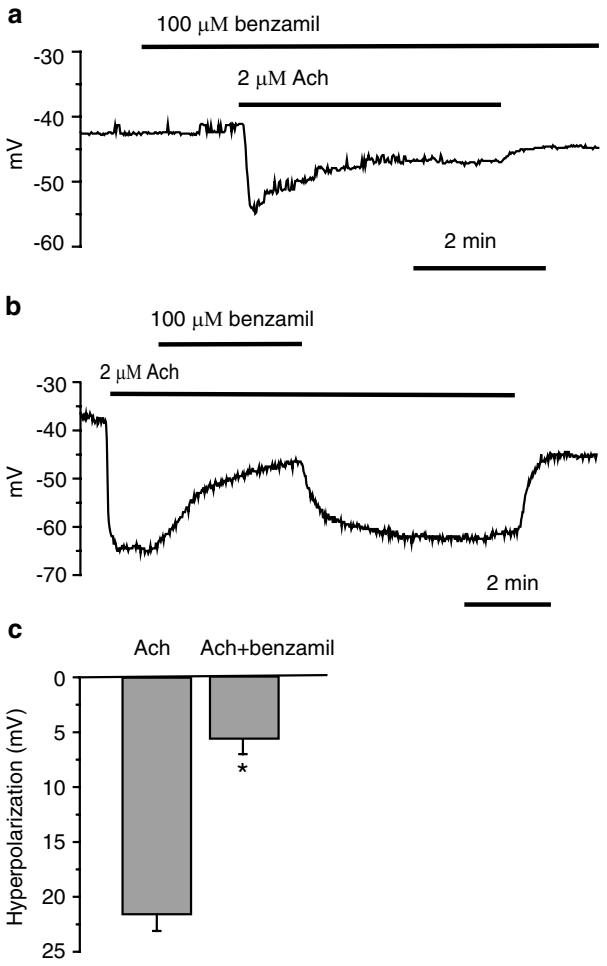

Figure 2 Effect of benzamil on Ach-induced sustained hyperpolarization of endothelial cells. (a) Transient hyperpolarization to application of 2 μM Ach in the presence of 100 μM benzamil. (b) Inhibition of sustained hyperpolarization to Ach by benzamil application during the plateau phase of hyperpolarization. (c) Effect of benzamil on Ach-induced sustained endothelial hyperpolarization.

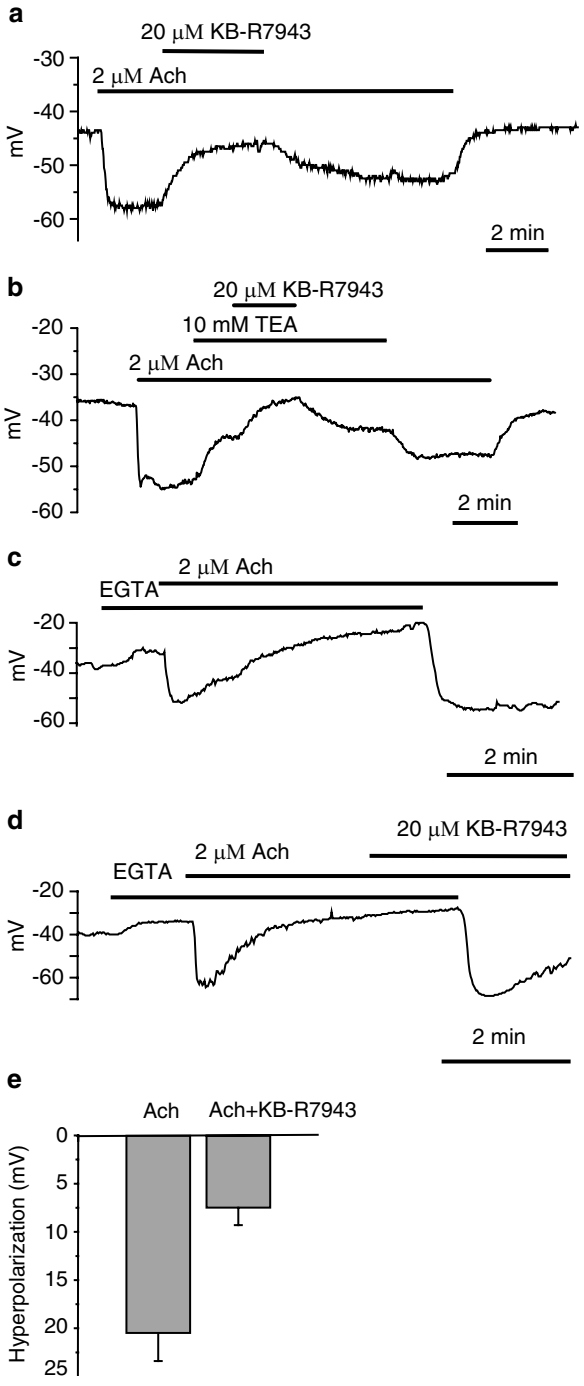

Figure 3 Effect of KB-R7943 on sustained Ach-induced hyperpolarization of endothelial cells. (a) Inhibition of sustained Ach-induced hyperpolarization of endothelial cells by 20 μM KB-R7943. (b) Inhibitory effect of KB-R7943 on Ach-induced hyperpolarization of endothelial cells in the presence of 10 mM TEA. Addition of 20 μM KB-R7943 further depolarized endothelium. (c, d) Effect of KB-R7943 on the membrane potential fluctuations caused by Ca^{2+} addition after the strip was exposed to Ach in Ca^{2+}-free solution, (c) Ca^{2+} was added in the absence of KB-R7943 and (d) Ca^{2+} was added in the presence of 20 μM KB-R7943. Note that KB-R7943 does not affect the amplitude of the hyperpolarization caused by Ca^{2+} influx, but accelerates the decay of the hyperpolarization. (e) Effect of KB-R7943 (20 μM) on Ach-induced sustained endothelial hyperpolarization.

The amplitude of hyperpolarization in these experiments decreased from 20.5 ± 2.9 to 7.5 ± 1.8 mV.

In an attempt to dissect the direct electrogenic role of NCX in hyperpolarization and the secondary role of stimulated K_{Ca}, the sensitivity of the plateau phase of hyperpolarization to tetraethylammonium (TEA), a blocker of large-conductance K_{Ca}, was tested. TEA at 10 mM has been reported to block completely large-conductance K_{Ca} in vascular endothelium (Demirel *et al*., 1994; Nilius & Droogmans, 2001). TEA at 10 mM depolarized endothelial cells by 6.0 ± 0.6 mV ($n = 5$). TEA was found to attenuate the plateau of Ach-induced hyperpolarization significantly. Thus, in these experiments, application of 2 μM Ach hyperpolarized endothelial cells by 17.9 ± 1.5 mV from the averaged resting value of -41.0 ± 2.5 mV. TEA (10 mM) administration in the continuous presence of Ach decreased the hyperpolarization by 12.9 ± 0.9 mV ($n = 10$). Subsequent addition of 20 μM KB-R7943 further depolarized endothelial cells by 4.8 ± 1.1 mV ($n = 4$) (Figure 3b).

To dissect the effect of blockade of reversed NCX on endothelial hyperpolarization evoked specifically by Ca^{2+} influx, a typical Ca^{2+} addition protocol was employed. As has been shown earlier, Ach administration in Ca^{2+}-free solution resulted in a transient hyperpolarization with no plateau (Figure 3c), reflecting stimulation of K_{Ca} by released intracellular Ca^{2+}. After the membrane potential returned to a baseline, Ca^{2+} was added to the Ach-containing bath solution, causing a maintained hyperpolarization, reflecting stimulated Ca^{2+} entry from extracellular space ($n = 5$). Introduction of 20 μM KB-R7943 to Ca^{2+}-free medium before Ca^{2+} addition was without effect ($n = 4$) (Figure 3d). However, the decay of the hyperpolarization caused by Ca^{2+} addition was accelerated in the presence of KB-R7943 ($n = 3$), while the amplitude was not affected. Thus, two structurally different blockers of NCX, benzamil and KB-R7943, exerted similar inhibitory effect on the sustained Ach-induced hyperpolarization of endothelial cells from intact rat aorta, suggesting that operation of NCX in reverse is a prerequisite for sustained endothelial hyperpolarization.

Role of extracellular Na^+ in endothelial hyperpolarization

The primary cause of activation of reversed NCX during simulation of endothelial cells by Ach must be a pronounced increase in $[Na^+]_i$. Under these conditions, the reversed NCX would over-ride the forward mode that occurs due to increased $[Ca^{2+}]_i$ as a result of Ca^{2+} release from intracellular stores and Ca^{2+} entry *via* store-operated channels. Therefore, substantial amount of Na^+ must be entering endothelial cells following Ach administration. To estimate the role of Na^+ influx in activation of the reversed NCX, external Na^+ was substituted by NMDG during Ach-induced hyperpolarization. These experiments were performed in HEPES-buffered solution to eliminate the electrogenic effect of sodium bicarbonate cotransporter present in endothelial cells. Ach hyperpolarized the endothelium from -45.1 ± 2.9 mV to a plateau level of -61.8 ± 2.7 mV ($n = 8$). Removal of external Na^+ during the plateau phase of hyperpolarization reversibly decreased the hyperpolarization to -54.2 ± 1.9 mV ($n = 8$) (Figure 4a). In five out of eight recordings, the initial response to removal of

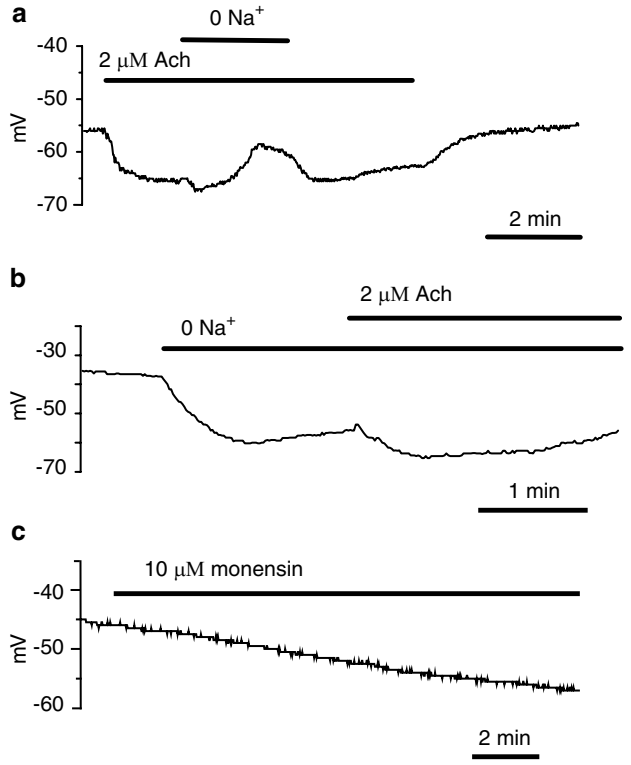

Figure 4 Dependence of endothelial hyperpolarization on Na^+ gradient. (a) Effect of external Na^+ withdrawal during Ach exposure on endothelial membrane potential fluctuations. (b) Effect of external Na^+ withdrawal before Ach exposure on endothelial membrane potential fluctuations. (c) Effect of Na^+ loading by monensin on endothelial membrane potential.

external Na^+ was a transient rise in the membrane potential with an amplitude of several mV. This rise apparently indicates a transient period of further stimulation of reversed NCX during which the Na^+ gradient is reversed before depletion of internal Na^+.

In order to further examine the role of extracellular Na^+ in Ach-induced hyperpolarization, Na^+ withdrawal was performed just before the application of Ach. Under these conditions, stimulation of the reversed NCX caused by Na^+ removal would deplete internal Na^+ before the store-operated Ca^{2+} entry is initiated, and an influx of Na^+ would be eliminated during stimulation by Ach. Removal of Na^+ from the bath solution evoked a hyperpolarization of endothelial cells by 13.8 ± 3.1 mV followed by a slow return to the resting potential. This is consistent with the reversal of NCX. Subsequent Ach administration during this initial hyperpolarization in Na^+-free solution led to a significantly smaller hyperpolarization (5.3 ± 1.8 mV, $n=4$) compared with the control response (16.7 ± 2.6 mV, $n=8$) (Figure 4b).

To verify whether an increase in internal Na^+ can produce endothelial hyperpolarization, the vessel strips were exposed to Na^+ ionophore monensin (10 μM) in Ca^{2+}-containing solution. Monensin evoked a slowly developing hyperpolarization of endothelial cells by 10.3 ± 0.7 mV ($n=3$). This observation is in line with the operation of NCX in reversed mode following Na^+ loading (Figure 4c).

Evaluation of the role of myoendothelial gap junctions in benzamil-mediated inhibition of sustained endothelial hyperpolarization

Since endothelial and smooth muscle cells are electrically coupled (Bény & Pacicca, 1994; Marchenko & Sage, 1994b), the possibility exists that inhibitory effect of the NCX blockers on Ach-induced endothelial hyperpolarization is accounted for by transmission of the response originated in smooth muscle cells to endothelial cells *via* myoendothelial gap junctions. To elucidate the role of gap junctions in the inhibition of endothelial hyperpolarization caused by benzamil, initially the putative gap junction inhibitor 18α-GA was tested. It was reported that GA derivatives reversibly block myoendothelial gap junctional communications within few minutes (Davidson & Baumgarten, 1988; Yamomoto *et al*., 1998) in some vascular beds, while having no appreciable effect on others (Tare *et al*., 2002). 18α-GA in concentrations up to 50 μM had no significant effect on the resting membrane potential of endothelial cells and on the amplitude of Ach-induced hyperpolarization (21.0 ± 1.1 mV, in the presence of 18α-GA, $n=7$, and 19.8 ± 1.2 mV in control, $n=33$). However, in the presence of 18α-GA, the inhibitory effect of benzamil on Ach-induced delayed hyperpolarization appeared to become significantly ($P<0.005$) blunted (5.8 ± 1.0 mV, $n=5$) compared with the control response (15.2 ± 1.7 mV, $n=15$) (Figure 5a). However, phenylephrine that acts directly on

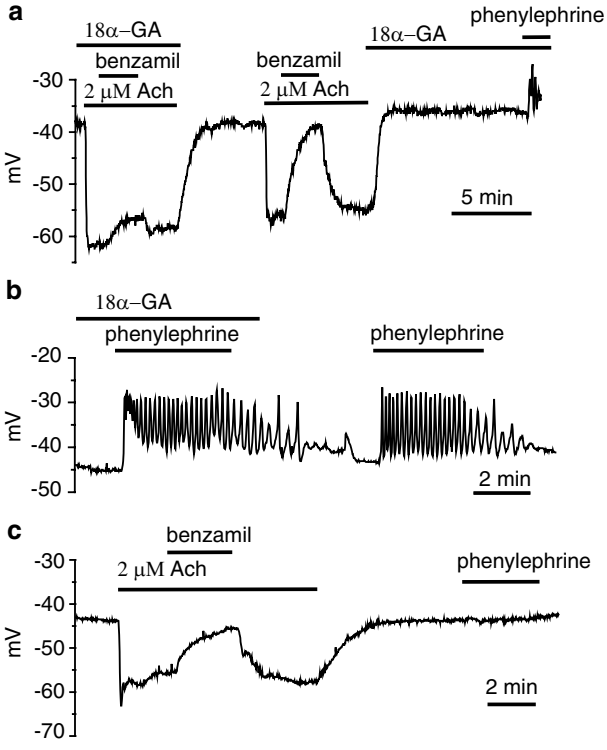

Figure 5 Evaluation of the role of gap junctions in inhibitory effect of benzamil on endothelial sustained hyperpolarization to Ach. (a) 18α-GA (20 μM) attenuates the effect of benzamil on Ach-induced sustained hyperpolarization. (b) 18α-GA (20 μM) does not inhibit the phenylephrine-induced oscillations in endothelial membrane potential. (c) Effect of 100 μM benzamil on Ach-induced hyperpolarization of mechanically isolated endothelium.

α1-adrenoreceptors of smooth muscle cells still caused oscillations in endothelial membrane potential in the presence of 20 μM 18α-GA (Figure 5a). 18α-GA (20 μM) has been found to affect neither amplitude nor frequency of phenylephrine-induced oscillations in endothelial membrane potential in rat aorta (Figure 5b). Such observations could be accounted for by functionally active electrical communications between smooth muscle cells and endothelial cells despite the presence of 18α-GA. These experiments clearly demonstrate that although 18α-GA attenuates the inhibitory effect of benzamil on Ach-induced endothelial hyperpolarization, this effect is not attributed to the inhibition of myoendothelial electrical communications.

Another way to rule out a possible participation of smooth muscles in the inhibitory effect of the NCX blockers on endothelial sustained hyperpolarization is to separate mechanically endothelial and smooth muscle layers. As has been described previously (Marchenko & Sage, 1994b), intima with endothelium can be removed from smooth muscles and Ach induces a typical response in such preparations, while electrical responses evoked by vasoconstrictors require the presence of smooth muscles. When recordings were commenced from mechanically isolated endothelium, 100 μM benzamil also inhibited Ach-induced hyperpolarization ($n=6$). Phenylephrine (1 μM) produced no effect on endothelial membrane potential in these preparations, which is consistent with the absence of smooth muscles (Figure 5c).

Discussion

In the present study, the sensitivity of Ach-induced sustained hyperpolarization to the blockers of NCX and extracellular Na^+ withdrawal was assessed in intact endothelial cells by using the perforated patch-clamp technique. This approach provides a substantial advantage in investigating the physiological role of NCX, since it allows physiological fluctuations of intracellular Na^+, Ca^{2+} and pH during stimulation of electrically coupled cells. Ach is known to exert its effect on endothelial membrane potential *via* stimulation of phospholipase C and inositol (1,4,5)-triphosphate (IP_3) formation (Marchenko & Sage, 1994a). IP_3 binding to specific receptors on endoplasmic reticulum is known to induce Ca^{2+} release from internal IP_3-sensitive stores, which in turn activates store-operated Ca^{2+} entry. $[Ca^{2+}]_{sp}$ rise activates K_{Ca}, resulting in endothelial hyperpolarization, which increases the electrochemical gradient for Ca^{2+} and facilitates the store-operated Ca^{2+} entry into endothelial cells. In many nonexcitable cells, including endothelial cells, the store-operated capacitative Ca^{2+} entry is considered the main mechanism responsible for agonist-induced Ca^{2+} entry. Ca^{2+} entry into endothelial cells stimulated by agonists is believed not to be mediated by the NCX mechanism (Schilling *et al*., 1988; Goto *et al*., 1996; Domotor *et al*., 1999; Sedova & Blatter, 1999; Moccia *et al*., 2002).

Store-operated channels are known to be permeable to Na^+ as well as Ca^{2+} (Arnon *et al*., 2000; Nilius & Droogmans, 2001), suggesting that activation of this pathway provides substantial Na^+ influx, which is significantly facilitated by membrane hyperpolarization. Moreover, endothelium-dependent vasodilators are known to cause intracellular alkalinization due to stimulation of Na^+/H^+ exchanger (Fleming *et al*., 1994). The latter, therefore, may represent another pathway for Na^+ entry following stimulation of endothelial cells. It is reasonable, therefore, to suggest that restoration of the sodium gradient would require activation of the Na^+ extrusion mechanisms. The idea that reversed NCX might be responsible for the plateau phase of agonist-induced increase in $[Ca^{2+}]_i$ in endothelial cells was first raised by Cannell & Sage (1989). However, bradykinin-stimulated increase in $[Ca^{2+}]_i$ in cultured endothelial cells (Schilling *et al*., 1988; Laskey *et al*., 1990) was not affected by isotonic substitution of external Na^+ with NMDG or Li^+, suggesting that NCX is not involved in Ca^{2+} entry during stimulation of endothelial cells by endothelium-dependent vasodilators.

Since there are fundamental differences between intact and cultured endothelial cells (Marchenko & Sage, 1993; Li & van Breemen, 1995), a possible role of NCX in mediating the stimulated Ca^{2+} entry into intact endothelial cells was verified in the present study using an electrophysiological approach. Dependence of the sustained hyperpolarization in response to Ach on extracellular Ca^{2+} entry observed in the present study supports the general notion that biphasic hyperpolarization evoked by endothelial agonists mirrors a biphasic elevation in $[Ca^{2+}]_i$ due to activation of K_{Ca} (Busse *et al*., 1988; Chen & Cheung, 1992; Bény & Pacicca, 1994; Usachev *et al*., 1995; Marchenko & Sage, 1996), the initial hyperpolarization being attributable to release of internal Ca^{2+} from IP_3-sensitive stores, while the sustained component of hyperpolarization is attributable to influx of extracellular Ca^{2+}.

Here an experimental evidence is provided that selective and structurally unrelated organic blockers of NCX, benzamil (inhibits both forward and reversed NCX) and KB-R7946 (inhibits reversed NCX), significantly inhibit the sustained phase of Ach-induced hyperpolarization of rat aortic endothelial cells. This finding suggests that the reversed mode of NCX mediates extracellular Ca^{2+} entry following stimulation of endothelial cells by Ach and is consistent with the earlier observations that NCX is involved in endothelium-dependent relaxation to Ach (Winquist *et al*., 1985; Schoeffter & Miller, 1986; Schneider *et al*., 2002). TEA, which preferentially blocks the large-conductance K_{Ca} (Demirel *et al*., 1994; Nilius & Droogmans, 2001), was also effective in inhibiting the sustained component of Ach-induced hyperpolarization. The large-conductance K_{Ca} have been demonstrated previously in intact aortic endothelium (Rusko *et al*., 1992; Demirel *et al*., 1994; Papassotiriou *et al*., 2000). TEA at 5 mM was reported to inhibit the Ach-induced $[Ca^{2+}]_i$ increase in native endothelium from rabbit aorta (Demirel *et al*., 1994) and Ach-induced hyperpolarization of endothelial cells from guinea pig coronary artery (Chen & Cheung, 1992). Thus, the observed sensitivity of endothelial hyperpolarization to TEA is consistent with the results obtained by other workers, who demonstrated participation of K_{Ca} in endothelial hyperpolarization to Ach. The potential involvement of SK_{Ca} in Ach-evoked hyperpolarization of endothelial cells from rat aorta had been ruled out previously by the lack of effect of SK_{Ca} channel blocker apamin on the hyperpolarization induced by Ach (Marchenko & Sage, 1996). However, intermediate-conductance K_{Ca} channels sensitive to high concentrations of charybdotoxin have been reported to participate in Ach-induced hyperpolarization in the same preparation (Marchenko & Sage, 1996). The present study was not aimed to identify precisely the K_{Ca} channel type responsible for the

hyperpolarization evoked by rise in $[Ca^{2+}]_{sp}$; therefore, the toxin sensitivity was not determined in additional experiments. The direct electrogenic effect of NCX, estimated by reaction on KB-R7943 addition in the presence of TEA, appeared to be ~ 5 mV in the present study. These results are consistent with the idea that reversed NCX may mediate hyperpolarization both directly, giving net efflux of positive charge, and indirectly due to secondary stimulation of K_{Ca} channels. However, it should be kept in mind that inhibition of hyperpolarization by K_{Ca} channel blockers inevitably decreases Ca^{2+} entry driven by electrochemical gradient and results in a fall in $[Ca^{2+}]_{sp}$. Since reversed NCX requires permissive intracellular Ca^{2+} that activates NCX in Ca^{2+} entry mode (Blaustein & Lederer, 1999), in the situation when sufficient Ca^{2+} is not supplied (inhibition of hyperpolarization), the reversed NCX might be relatively dormant. This feature of NCX might underlie the observations made by others that potent blockers of K_{Ca} channels may completely inhibit the hyperpolarizing responses of endothelial cells, and contribution of reversed NCX is not seen.

It has been shown that Cl^- channels may also contribute to membrane potential fluctuations induced by Ach in isolated endothelial cells (Voets *et al*., 1996; Wang & van Breemen, 1999). However, it is unlikely that they participate in long-lasting hyperpolarization observed in the present study. A maintained hyperpolarization is thought to be a signature of preparations in which the contribution of Cl^- channels is minimal (Wang & van Breemen, 1999); in these preparations, the hyperpolarizing response is mediated mainly by K_{Ca}. In contrast, preparations, in which contribution of Cl^- channels to membrane depolarization is noticeable, respond with the short-lived hyperpolarization (Busse *et al*., 1988; Wang & van Breemen, 1999). Moreover, blockade of the Cl^- channels leads to membrane hyperpolarization (Voets *et al*., 1996), which makes highly unlikely that Cl^- channels underlie the observed electrogenic effect of blockade of NCX.

The observed inhibition of Ach-evoked sustained hyperpolarization by selective NCX blockers suggests that Na^+ must increase substantially to permit operation of NCX in Ca^{2+} entry mode. In an attempt to inhibit the reversed NCX nonpharmacologically, by depletion of internal Na^+, external Na^+ was substituted with NMDG during the plateau phase of hyperpolarization. This manipulation had a similar effect on endothelial sustained hyperpolarization as administration of Ni^{2+}, benzamil and KB-R7943, known blockers of NCX. In the majority of recordings, the hyperpolarization initially increased transiently before being inhibited. This transient hyperpolarization may indicate further stimulation of reversed NCX during which the internal Na^+ is depleted. Inhibition of reversed NCX as a result of depletion of intracellular Na^+ is consistent with the previously reported observation that L-citrullin formation is attenuated in Na^+-free solution in thapsigargin-depleted cells despite the lack of effect on overall $[Ca^{2+}]_i$ (Teubl *et al*., 1999). The role of internal Na^+ rise in the maintained Ach-induced hyperpolarization was further supported by experiments with Na^+ substitution just before Ach application. External Na^+ removal is expected to create a large outward Na^+ gradient, significantly increasing the driving force for Ca^{2+} entry in cells whose plasma membrane contains a functional NCX. The energetics of the exchanger predicts that the driving force for the reversed mode of NCX under these conditions is unrestricted. Endothelial cells were hyperpolarized in the present study by 14 mV following Na^+ removal as expected for stimulation of reversed NCX. Further Ach administration produced much smaller hyperpolarization than in controls. A possible explanation for this observation is as follows: when Ach was added some minutes after the cells were in Na^+-free solution, $[Na^+]_i$ most likely was significantly lowered and Na^+ entry was prevented, which would tend to decrease the expected large hyperpolarization to Ach. Furthermore, direct Na^+ loading with monensin, which is a widely used tool aimed to activate operation of NCX in reverse, hyperpolarized endothelium in the present study. Previously, it was shown that the exchanger is able to mediate Ca^{2+} influx into endothelial cells only following exposure to low Na^+ extracellular solution (Sage *et al*., 1991; Domotor *et al*., 1999; Schneider *et al*., 2002), that is, under extreme experimental, but not physiological, conditions. Recently, however, it has been demonstrated that the exchanger is able to mediate Ca^{2+} influx in response to H_2O_2 exposure in freshly dissociated coronary artery endothelial cells (Bowles *et al*., 2001). Similar to the observation of the present study, the plateau phase of $[Ca^{2+}]_i$ increase caused by H_2O_2 in the study of Bowles *et al*. (2001) was reversibly inhibited by low Na^+ external solution, demonstrating the crucial role of stimulated Na^+ entry in stimulation of reversed NCX. Taken together, these experiments support the view that Na^+ loading is an essential step in generation of Ach-induced response *via* stimulation of the reversed NCX in intact endothelium.

Membrane hyperpolarization is known to favor the forward mode of the exchanger. However, the driving force calculations suggest that at conditions close to experimental during hyperpolarization ($Vm = -65$ mV, $[Ca^{2+}]_i = 450$ nM (Usachev *et al*., 1995; Huang *et al*., 2000), $[Na^+]_o = 145$ mM and $[Ca^{2+}]_o = 1.8$ mM), the exchanger will reverse when $[Na^+]_i$ reaches 23 mM. The reversal potential of the exchanger at these conditions is -71 mV. Taking into account that the reported resting values for $[Na^+]_i$ are in the range of 19.7–24 mM (Hansen *et al*., 1991; Cutaia *et al*., 1996) and that substantial amount of Na^+ is entering endothelial cells during cell stimulation, it would be reasonable to predict that $[Na^+]_i$ can reach much higher values during Ach exposure than the reported resting values for $[Na^+]_i$ of $\sim$20–24 mM, stimulating thus reversed NCX, when cells are hyperpolarized. Experiments with blockade of K_{Ca} by TEA demonstrated that the hyperpolarizing effect caused by operation of NCX in reversed mode is mostly mediated by the secondary activation of K_{Ca} rather than by direct electrogenic effect of NCX. Therefore, even a small rate of reversed NCX is expected to be sufficient to produce a significant hyperpolarizing effect. This, in turn, would support sustained Ca^{2+} entry driven by electrochemical gradient that is necessary for eNOS stimulation.

Several pathways are described in endothelial cells through which Na^+ can enter the cytosol after Ach exposure. The first candidate is a nonselective channel that is permeable for Na^+ and Ca^{2+}. Unloading of internal IP_3-sensitive Ca^{2+} stores has been reported to activate nonselective channels with permeation ratio $P_{Ca}:P_{Na}$ between 0.03 and 2 (Nilius & Droogmans, 2001). Amiloride-sensitive Na^+ channels that have been described in cultured endothelial cells derived from brain microvessels with a conductance of 23 pS for Na^+ and K^+ (Vigne *et al*., 1989) might also be involved; however, the presence of amiloride-sensitive channels in intact aortic

endothelium is questioned. Recently, a nonselective cationic channel has been described in intact endothelial cells from rat aorta with a conductance of 5.4 pS for Na^+ and K^+ (Marchenko, 2002). It is unclear, however, whether this channel is sensitive to amiloride. If so, it is possible that the site of action of benzamil, which is a derivative of amiloride, is both NCX and an Na^+-permeable channel. But blockade of Na^+-permeable channel (pathway) would rather tend to hyperpolarize the membrane if NCX is not operating in reverse. However, the opposite was observed. Therefore, possible selectivity of benzamil to this channel type would not exclude the operation of NCX in reverse. Moreover, KB-R7943, which is an isothiourea derivative, was found to have a similar inhibitory effect as benzamil on delayed hyperpolarization. The third pathway that may contribute to Na^+ loading is Na^+/H^+ exchanger. Activation of this antiporter, which mediates intracellular alkalinization following agonist and mechanical stimulation, has been well documented (Ghigo *et al*., 1988; Fleming *et al*., 1994). Moreover, inhibition of Na^+/H^+ exchanger with EIPA (Ghigo *et al*., 1988) and with HOE 694 (Fleming *et al*., 1994) was reported to prevent Ca^{2+} entry into human endothelial cells stimulated by thrombin and bradykinin, respectively. However, it is unclear at present whether this effect is mediated by reversed NCX as a result of inhibition of Na^+ entry.

Neither benzamil nor KB-R7943 significantly affected endothelial resting membrane potential in the present study. However, the exchanger energetics favors the reversed mode of NCX at $Vm = -45$ mV, assuming that resting $[Na^+]_i$ is 24 mM (Hansen *et al*., 1991; Cutaia *et al*., 1996) and resting $[Ca^{2+}]_i$ is 95 nM (Usachev *et al*., 1995; Huang *et al*., 2000). Moreover, the driving force for reversed mode NCX function is apparently much larger at rest than after Ach addition. Under these conditions, benzamil and KB-R7943 are expected to depolarize the cells significantly, if reversed NCX is active. However, it is known that internal Ca^{2+} is required for activation of Ca^{2+} entry mode of the exchanger (Blaustein & Lederer, 1999). As has been suggested previously (Li & van Breemen, 1995), reversed NCX might be relatively inactive in the resting endothelial cells until $[Ca^{2+}]_i$ is raised to a certain level. A rise in $[Ca^{2+}]_{sp}$ and resulting initial hyperpolarization, therefore, might be a triggering step in activation of NCX. This suggestion is further supported by the observation that $[Ca^{2+}]_i$ buffering by pretreatment with BAPTA-AM greatly reduced or completely abolished hyperpolarization to Ach (personal observation; Marchenko & Sage, 1994a), suggesting that clamping of internal Ca^{2+} prevents the reversed NCX from activation. On the other hand, results of the present study demonstrate that the amplitude of hyperpolarization evoked by Ca^{2+} restoration in the presence of Ach was not affected by KB-R7943. However, KB-R7943 markedly shortened the hyperpolarization. This would suggest that initial part of Ca^{2+} entry (required for activation of NCX?) is not mediated by NCX, but NCX is activated following Ca^{2+} entry and mediates delayed Ca^{2+} influx.

The present study also evaluated the role of gap junctions in inhibition of endothelial sustained hyperpolarization by the blockers of NCX. These experiments were aimed to exclude the possibility that the inhibitory effect of the NCX blockers on endothelial hyperpolarization results from signal transmission originated in smooth muscle cells. In the first set of experiments, 18α-GA was used as a tool to disrupt myoendothelial electrical communications. 18α-GA was chosen because it was reported to be a more potent and specific form of GA derivative that blocks gap junctions (Davidson & Baumgarten, 1988). Although in the present study 18α-GA significantly attenuated the inhibitory effect of benzamil on sustained hyperpolarization, phenylephrine-induced oscillations in endothelial membrane potential were still observed in the presence of 18α-GA. A number of vasoconstrictors including phenylephrine are known to act on receptors on smooth muscles and induce depolarization and oscillations in the membrane potential of intact endothelium, which is accounted for by signal transmission from smooth muscle cells (Marchenko & Sage, 1994b). 18α-GA in the present study had no appreciable effect on the amplitude and frequency of phenylephrine-induced oscillations in endothelial membrane potential. These experiments clearly demonstrate that attenuation of the inhibitory effect of benzamil on endothelial sustained hyperpolarization by 18α-GA is not attributed to the inhibition of gap junctions. The observed limited ability of 18α-GA to block gap junctions in rat aorta is consistent with the recently reported observation made on guinea pig coronary and rat mesenteric arteries (Tare *et al*., 2002).

To assess directly the role of smooth muscles in the inhibition of endothelial hyperpolarization by the NCX blockers, recordings were commenced from endothelium that was mechanically isolated from smooth muscle. In such preparations, Ach has been reported to evoke a typical response, while phenylephrine is without effect. In these experiments, the inhibitory effect of benzamil on delayed hyperpolarization was still observed, confirming that the benzamil-evoked response does not require the presence of smooth muscle and is generated within the endothelium itself.

In conclusion, the present study suggests that the reversed NCX of intact endothelial cells from rat aorta contributes to sustained hyperpolarization following Ach administration both directly and indirectly due to secondary stimulation of K_{Ca}. The latter feature enables NCX not only to mediate but also to control Ca^{2+} entry driven by electrochemical gradient. Stimulating the reversed mode of NCX, Na^+ loading is an essential step in generation of sustained endothelial hyperpolarization in response to Ach. The inhibitory effect of NCX blockers on Ach-induced endothelial hyperpolarization is not mediated by gap junction-mediated transmission of the response originated in smooth muscle cells to endothelial cells. These findings suggest that NCX is an important modulator of endothelial membrane potential during stimulation by Ach and both mediator and modulator of stimulated Ca^{2+} entry.

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