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RESEARCH PAPER

Na^+-K^+-ATPase is involved in the sustained ACh-induced hyperpolarization of endothelial cells from rat aorta

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Background and purpose. Inhibition of Na^+-K^+-ATPase is known to attenuate endothelium-dependent relaxation in many arteries. The purpose of this study was to evaluate the role of Na^+-K^+-ATPase in the regulation of endothelial membrane potential at rest and during stimulation by ACh.
Experimental approach. Membrane potential was recorded from the endothelium of rat aorta using the perforated patch-clamp technique.
Key results. Superfusion with K^+-free solution produced a depolarization of about 11 mV from the resting value of -42.9 $\pm$ 0.9 mV. Reintroduction of 4.7 mM K^+ transiently hyperpolarized endothelial cells to -52.4 ± 1.8 mV and the membrane potential recovered within 10 min. Ouabain 500 μM depolarized endothelium by about 11 mV and inhibited the hyperpolarization induced by K^+ reintroduction into the K^+-free solution. However, 500 nM ouabain did not affect the resting membrane potential or the hyperpolarization induced by K^+ reintroduction. Pre-exposure to ouabain 500 μM, but not 500 nM, attenuated the sustained component of hyperpolarization to ACh without affecting the amplitude of the transient peak hyperpolarization. In K^+-free solution, the amplitude of peak hyperpolarization to ACh was increased, while the sustained component of hyperpolarization was attenuated.
Conclusions and Implications. These results indicate that electrogenic Na^+-K^+-ATPase partially contributes to the sustained hyperpolarization of endothelial cells from rat aorta in response to ACh. They also suggest that the α1, but not α2 or α3 isoforms, is involved in ACh-mediated hyperpolarization.
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Keywords: rat aorta; endothelium; membrane potential; Na pump

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; EDHF, endothelium-derived hyperpolarizing factor; EGTA, ethyleneglycol tetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethyl-sulphonic acid; $[Na^+]_i$, intracellular Na^+ concentration; L-NMMA-, N^G-monomethyl-L-arginine monoacetate; NO, nitric oxide

Introduction

Na^+-K^+-ATPase is known to have an essential role in the regulation of vascular tone (Marin and Redondo, 1999). Inhibition of Na^+-K^+-ATPase by cardiac glycosides or K^+-free solution has been shown to attenuate or abolish endothelium-dependent relaxation in a number of arteries, including aorta (Rapoport *et al.*, 1985; Hirano *et al.*, 1992; Lee *et al.*, 1992; Edwards *et al.*, 1998; Ferrer *et al.*, 1999; Van de Voorde and Vanheel, 2000; Jiang and Dusting, 2001). By stimulating the release of nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF), ACh induces endothelium-dependent smooth muscle hyperpolarization that then causes endothelium-dependent relaxation. Suppression of endothelium-dependent hyperpolarization by ouabain in a number of vascular preparations, such as canine coronary artery (Feletou and Vanhoutte, 1988), porcine coronary artery (Olanrewaju *et al.*, 1997) and rabbit cerebral artery (Brayden, 1990), has been attributed to inhibition of the smooth muscle Na^+-K^+-ATPase. Thus, it has been proposed that during stimulation by endothelium-dependent vasodilators, the smooth muscle Na^+-K^+-ATPase is stimulated by endothelium-derived NO (Rapoport *et al.*, 1985; Gupta *et al.*, 1994) or by K^+ released from endothelial cells (Edwards *et al.*, 1998).

Although the triggering role of endothelial hyperpolarization in endothelium-dependent smooth muscle hyperpolarization is widely accepted, the role of Na^+-K^+-ATPase in endothelial electrical responses to endothelium-dependent vasodilators has only been evaluated in a few studies (Chen

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and Cheung, 1992; Edwards *et al*., 1998; White and Hiley, 2000; Jiang *et al*., 2005). In these studies, only the peak hyperpolarization, which reflects Ca^{2+} release from internal stores, has been assessed and found not to be sensitive to ouabain. However, the sustained endothelial hyperpolarization, which facilitates Ca^{2+} influx into endothelial cells required for Ca^{2+}-dependent NO synthesis and EDHF-type responses (Fukao *et al*., 1997; Tomioka *et al*., 2001; Ungvari *et al*., 2002) has not been fully characterized. Moreover, direct transmission of endothelial hyperpolarization to adjacent smooth muscle cells via gap junctions, a phenomenon which has been shown to underpin the EDHF-type response (Coleman *et al*., 2001), indicates the need to investigate further the mechanisms involved in the attenuation of endothelium-dependent relaxation by ouabain.

Recently, it has been shown that inhibition of the sodium–calcium exchanger (NCX) in rat aortic endothelial cells drastically inhibits the sustained endothelial hyperpolarization to ACh (Bondarenko, 2004), suggesting that activation of reverse NCX and possibly other Na^+ extrusion mechanisms are induced by the increased Na^+ influx. The ensuing rise in internal Na^+ may also stimulate the electrogenic Na^+-K^+-ATPase, thereby modulating the membrane hyperpolarization and, hence, the driving force for Ca^{2+} entry.

The experiments described here were designed to evaluate the contribution of the Na^+-K^+-ATPase to electrogenesis of endothelial cells in rat aorta, in resting conditions and during stimulation by ACh, particularly with regard to the time course of the response.

Methods

This investigation conforms to the guidelines of the Institutional Committee for Biomedical Research Ethics, the experimental procedures were carried out in accordance with the regulations of the UK Animals (Scientific Procedures) Act 1986.

Experiments were performed on 2–4 months old Wistar–Kyoto rats of either sex. Rats were killed by stunning followed by cervical dislocation. Thoracic aortae were excised and placed in bubbled (95% O_2 and 5% CO_2) Krebs 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 experiments commenced, the segments were cut open, and a strip was pinned to the rubber bottom of a 100 μl chamber and superfused with physiological saline solution at a rate of 0.5 ml min^{-1}. When K^+ -free solution was used, KCl was replaced by an equimolar amount of NaCl.

Membrane potential was recorded from intact aortic endothelial cells using the whole cell configuration of the patch-clamp technique in current clamp mode. Electrical contact with the cytosol was established using nystatin. Nystatin was dissolved in dimethyl sulphoxide, and the final nystatin concentration in the pipette solution was 200 μM. Patch pipettes, after being filled with a solution containing (mM) KCl 140, NaCl 10, ethyleneglycol tetraacetate (EGTA) 0.5, 4-(2-hydroxyethyl)-1-piperazineethyl-sulphonic acid (HEPES) 10, had a resistance of 5–7 MΩ. The pH of the pipette solution was adjusted to 7.2 by addition of KOH. Experiments were conducted at room temperature. Pharmacological agents were applied to the preparation by bath perfusion. Comparisons of endothelial hyperpolarization in response to ACh before and after Na^+-K^+-ATPase inhibition by ouabain or K^+-free solution were performed after continuous recordings had been successfully established. Quantitative data are expressed as means ± s.e. Statistical significance was evaluated by Student's *t*-test. *P*-values <0.05 were considered statistically significant.

Results

Effect of Na^+-K^+-ATPase inhibition on endothelial membrane potential

Superfusion of the vessel strip with K^+-free solution produced an endothelial depolarization of 11.7 ± 0.6 mV from a resting value of -42.9 ± 0.9 mV ($n=50$). Subsequent exposure to 100 μM benzamil, a NCX blocker, did not alter the membrane potential (from -28.1 ± 1.0 mV to -28.6 ± 1.4 mV, $n=7$) (Figure 1a). Addition of 4.7 mM K^+ to the bath solution transiently hyperpolarized endothelial cells from a mean value of -31.3 ± 1.9 mV in K^+-free solution to a peak level of -52.4 ± 1.8 mV ($n=14$). Then the membrane potential slowly returned towards a resting value. Five minutes after the peak, the membrane potential (-49.3 ± 1.8 mV, $n=14$) was still significantly ($P<0.05$) hyperpolarized compared to the resting level (-43.2 ± 1.6 mV, $n=14$) and 10 min after the peak, the membrane potential was still more negative (-46.4 ± 1.9 mV, $n=14$), but the difference was not statistically significant.

Superfusion of the vessel strip with a solution containing ouabain (500 μM; an inhibitor of Na^+-K^+-ATPase) at a concentration sufficient to block both α1 and α2 ion-transporting subunits, depolarized endothelial cells by 11.4 ± 0.9 mV from a resting value of -45.1 ± 1.5 mV ($n=24$) (Figure 1b). However, the membrane potential was not modified when 500 μM ouabain was administered in the absence of external K^+ ($n=3$). Addition of 4.7 mM K^+ in the presence of 500 μM ouabain produced only minor changes in the membrane potential ($n=3$) (Figure 1c), indicating effective inhibition of Na^+-K^+-ATPase by 500 μM ouabain. Ouabain withdrawal caused a gradual return of the membrane potential towards baseline.

In contrast, a low concentration of ouabain (500 nM), which has been reported to block α2 and α3 isoforms of the Na^+-K^+-ATPase selectively (Blanco and Mercer, 1998), did not affect the resting membrane potential (from -45.9 ± 2.9 to -45.7 ± 2.8 mV, $n=6$) (Figure 1d). Essentially, ouabain at 500 nM was not effective at inhibiting the hyperpolarization induced by K^+ reintroduction into K^+-free solution ($n=3$) (Figure 1d).

Effect of Na^+-K^+-ATPase inhibition on ACh-induced endothelial hyperpolarization

Vasoactive agonists cause Na^+ entry into endothelial cells via a variety of pathways (Nilius and Droogmans, 2001).

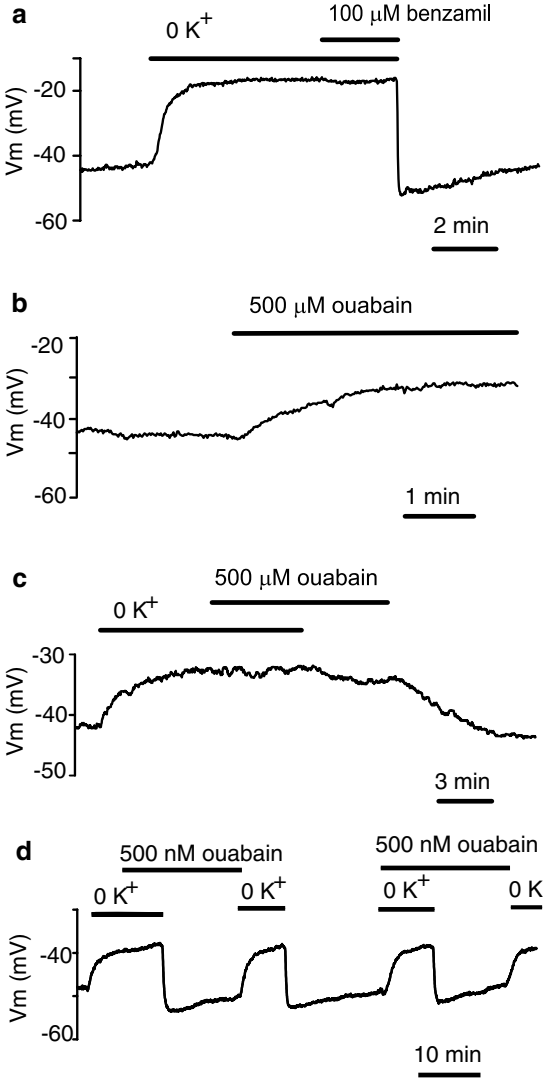

Figure 1 Modulation of endothelial membrane potential by Na^+-K^+-ATPase inhibition. (**a**) Effects of external K^+ withdrawal followed by 100 μM benzamil administration and K^+ reintroduction on the membrane potential. (**b**) Effect of 500 μM ouabain on endothelial membrane potential. (**c**) Lack of depolarizing effect of 500 μM ouabain in the absence of external K^+ and inhibitory effect of 500 μM ouabain on the hyperpolarization induced by K^+ reintroduction. (**d**) Lack of inhibitory effect of 500 nM ouabain on the hyperpolarization induced by K^+ reintroduction.

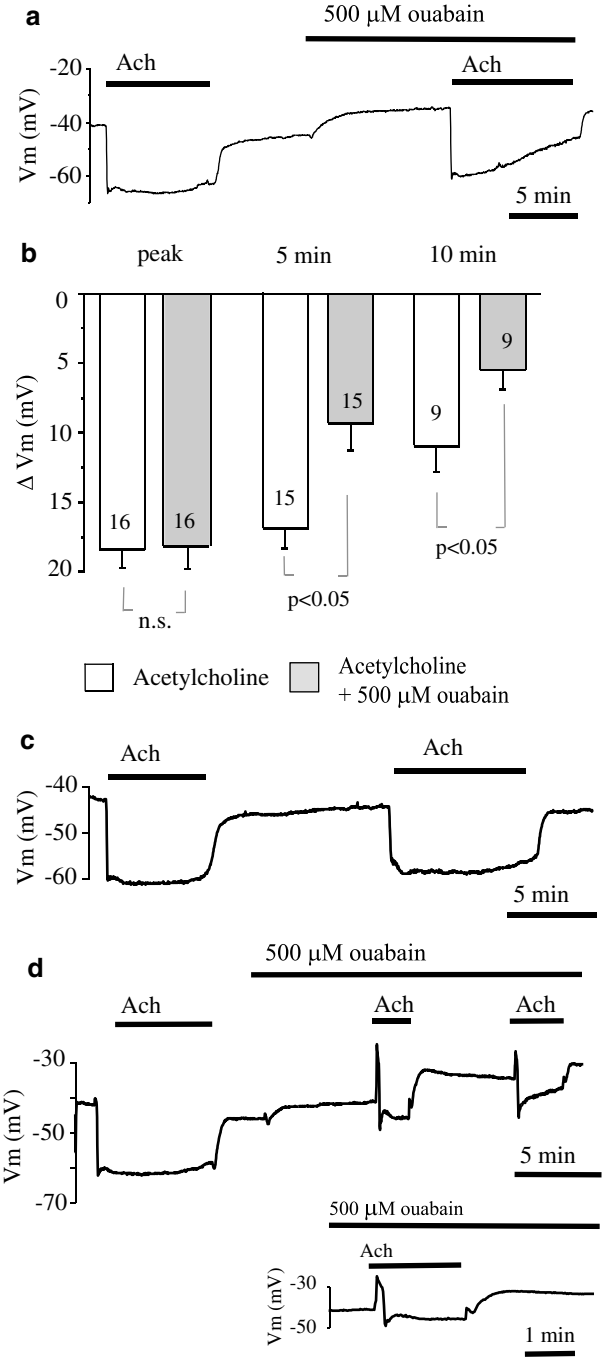

Figure 2 Effect of 500 μM ouabain on the hyperpolarization of endothelial cells from rat aorta produced by acetylcholine. (**a**) Original tracing showing the influence of 500 μM ouabain on the hyperpolarization of endothelial cells produced by 2 μM acetylcholine. (**b**) Graphical representation of the time dependency of the effect of 500 μM ouabain on the mean changes in membrane potential evoked by 2 μM acetylcholine. The changes in membrane potential were calculated as the difference between the membrane potential values before and after acetylcholine administration. Numbers associated with bar plot indicate the number of aorta preparations. (**c**) Representative record of repeated applications of 2 μM acetylcholine. (**d**) Original tracing showing attenuation of all phases of acetylcholine-evoked hyperpolarization by 500 μM ouabain that unmasked a transient depolarization phase in response to acetylcholine. For clarity, a portion of the trace has been expanded.

Conceivably, following ACh administration, the Na^+-K^+-ATPase of endothelial cells may be further stimulated, thus contributing to membrane hyperpolarization. To test this assumption, we compared endothelial electrical responses to ACh before and after Na^+-K^+-ATPase inhibition. ACh was applied at intervals not less than 10 min. During this period, a control bath solution was switched to either K^+-free or ouabain-containing solution.

ACh (2 μM) produced a transient peak hyperpolarization of 18.4 ± 1.3 mV ($n = 16$). This transient hyperpolarization was followed by a sustained hyperpolarization with a partial reduction in amplitude. In the presence of 500 μM ouabain, the membrane potential was significantly depolarized.

When ACh was again applied in the presence of 500 μM ouabain, the initial peak hyperpolarization (18.1±1.6 mV, $n=16$) was not altered and the membrane potential (−57.3±1.6 mV) attained during the peak hyperpolarization did not reach ($P<0.05$) the level attained in the absence of ouabain (−63.1±0.9 mV). However, as shown in Figure 2a, in the presence of ACh, the membrane potential recovered faster after inhibition of the Na^+-K^+-ATPase by 500 μM ouabain. Close inspection revealed that at 5 min after the peak, the amplitude of sustained hyperpolarization was significantly ($P<0.05$) attenuated by 500 μM ouabain (from 16.9±1.4 mV, $n=15$ to 9.3±2.0 mV, $n=15$). At 10 min, the hyperpolarization was further attenuated ($P<0.05$) by ouabain (from 10.9±1.9 mV, $n=9$ to 5.5±1.4 mV, $n=9$). This effect was not seen when repeated ACh applications were administered in the control Krebs solution ($n=5$) (Figure 2c). In one preparation, ouabain (500 μM) administration resulted in attenuation of all phases of the ACh-evoked hyperpolarization and the hyperpolarization was preceded by a transient depolarization of up to 15 mV (Figure 2d). In contrast, 500 nM ouabain failed to affect either the transient (from 18.3±2.1 mV, $n=6$ to 20.7±3.0 mV, $n=6$) or the sustained component of the hyperpolarization (at 5 min, from 15.9±1.9 mV, $n=6$ to 17.9±3.3 mV, $n=6$, and at 10 min, from 11.4±2.1 mV $n=6$ to 12.0±3.1 mV, $n=6$) (Figure 3).

When K^+-free solution was used to inhibit the Na^+-K^+-ATPase, the membrane potential was depolarised and the amplitude of peak hyperpolarization (33.1±2.8 mV, $n=16$) produced by 2 μM ACh was significantly ($P<0.05$) larger then that observed before omission of external K^+ (21.1±1.8 mV, $n=14$). At 5 min after the peak, the amplitude of sustained hyperpolarization to ACh in K^+-free solution (15.8±3.1 mV, $n=16$) was not significantly different from that obtained before omission of external K^+ (16.4±2.0 mV, $n=14$) (Figure 4). However, at 7 min after the peak, the amplitude of hyperpolarization in K^+-free solution (5.9±2.3 mV, $n=12$) decreased significantly ($P<0.05$) compared with the control response (14.1±3.4 mV, $n=7$). This difference was even more significant at 10 min after the peak. At this point, ACh-induced hyperpolarization in K^+-free solution completely decayed (the membrane potential values were −31.3±2.5 and −28.5±2.4 mV ($n=4$) before and after ACh administration, respectively), whereas in the control solution, the amplitude of hyperpolarization still amounted to 12.0±3.7 mV ($n=4$). These data demonstrate that Na^+-K^+-ATPase blockade with either 500 μM ouabain or omission of external K^+ attenuates the sustained hyperpolarization of endothelial cells induced by ACh.

We further sought to investigate whether Na^+-K^+-ATPase contributes to the hyperpolarization of endothelial cells produced independently of receptor stimulation. Ca^{2+} ionophores at low concentrations are thought to act selectively on Ca^{2+} storage sites without a significant direct increase in the Ca^{2+} permeability of the plasma membrane (Itoh *et al.*, 1985). This leads to a depletion of stored Ca^{2+}

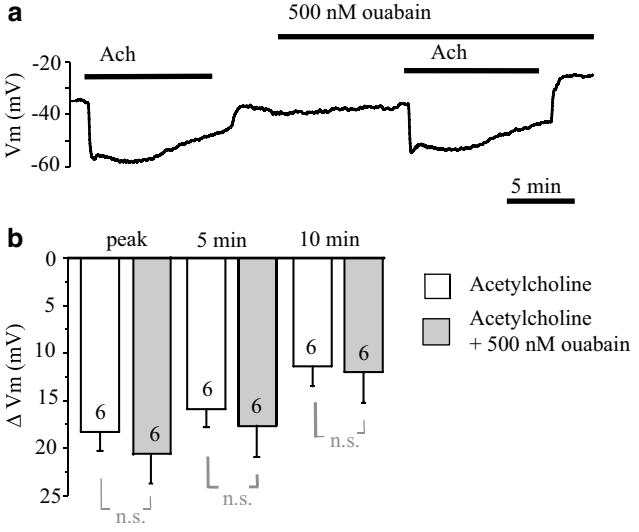

Figure 3 Influence of a low concentration of ouabain (500 nM) on the hyperpolarization of endothelial cells produced by acetylcholine. (**a**) Original tracing showing a lack of effect of 500 nM ouabain on the hyperpolarization of endothelial cells produced by 2 μM acetylcholine. (**b**) Graphical representation of the time dependency of the effect of 500 nM ouabain on the mean changes in membrane potential evoked by 2 μM acetylcholine. The changes in membrane potential were calculated as the difference between the membrane potential values before and after acetylcholine administration. Numbers associated with bar plot indicate the number of aorta preparations.

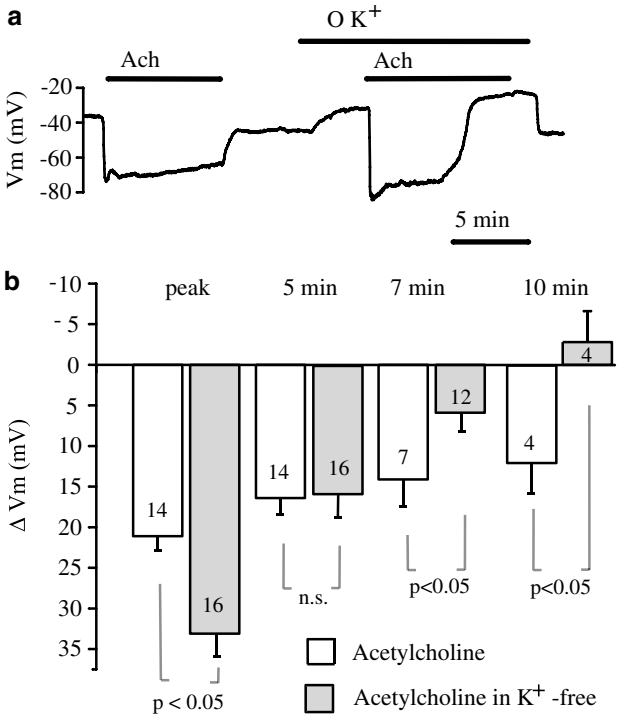

Figure 4 Effect of K^+-free solution on the hyperpolarization of endothelial cells from rat aorta produced by acetylcholine. (**a**) Original tracing showing the influence of external K^+ substitution on the resting membrane potential and acetylcholine-induced hyperpolarization of endothelial cells. (**b**) Graphical representation of the time dependency of the effect of K^+-free solution on the mean changes in membrane potential evoked by 2 μM acetylcholine. The changes in membrane potential were calculated as the difference between the membrane potential values before and after acetylcholine administration. Numbers associated with bar plot indicate the number of aorta preparations.

with subsequent activation of store-operated channels. Hence, to evoke this we used a low concentration of ionomycin. Superfusion of the vessel strip with ionomycin (0.6 μM) produced endothelial cell hyperpolarization followed by a recovery of the membrane potential that turned to a depolarization of several mV beyond the resting level (Figure 5). The initial hyperpolarization to ionomycin developed substantially slower than that produced by ACh. However, the membrane potential recovered faster. Ouabain 500 μM did not modify the amplitude of ionomycin-induced peak hyperpolarization (from 23.1 ± 3.4 mV, $n=7$ to 22.8 ± 2.8 mV, $n=5$). Although 500 μM ouabain slightly decreased the amplitude of ionomycin-evoked hyperpolarization at 5 min after the peak (from 13.6 ± 2.6 mV, $n=7$ to 10.6 ± 1.5 mV, $n=5$), the difference did not reach statistical significance. At 10 min, the hyperpolarization to ionomycin amounted to 2.9 ± 3.3 mV ($n=7$), whereas in the presence of ouabain the membrane response turned to a depolarization of $6.4 + 2.0$ mV ($n=5$, $P<0.05$) (Figure 5).

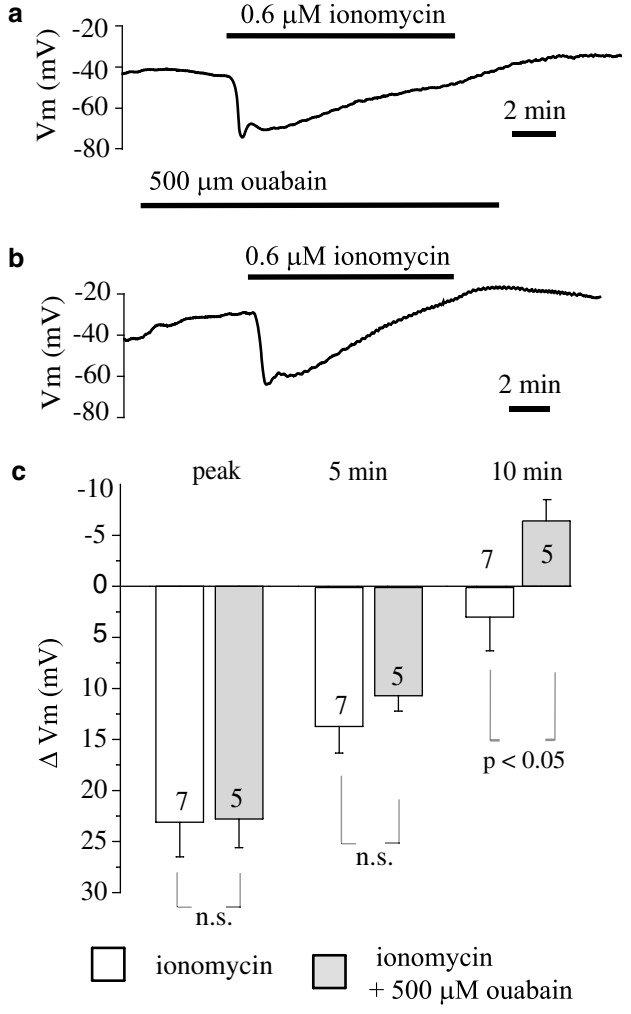

Figure 5 Influence of 500 μM ouabain on the hyperpolarization of endothelial cells from rat aorta produced by ionomycin. (**a**) Original tracing of an experiment showing the influence of ionomycin (0.6 μM) on the membrane potential of endothelial cells. (**b**) Original tracing of an experiment showing the influence of 500 μM ouabain on the hyperpolarization of endothelial cells produced 0.6 μM ionomycin. (**c**) Graphical representation of the time dependency of the effect of 500 μM ouabain on the mean changes in membrane potential evoked by 0.6 μM ionomycin. The changes in membrane potential were calculated as the difference between the membrane potential values before and after ionomycin administration. Numbers associated with bar plot indicate the number of aorta preparations.

The inhibitory effect of ouabain and K^+-free solution on the sustained component of endothelial hyperpolarization may indicate that the Na^+-K^+-ATPase promotes a sustained endothelial hyperpolarization in response to ACh. Apart from the intracellular Na^+ concentration ($[Na^+]_i$) rise, an increase in K^+ concentration close to the endothelial cell membrane owing to activation of calcium-dependent potassium channels may also stimulate the endothelial Na^+-K^+-ATPase. To test this possibility, the extracellular K^+ concentration was increased from 4.7 to 10.7 mM. This increase consistently depolarized endothelial cells by 2.2 ± 0.2 mV ($n=5$). Conceivably, if extracellular K^+ stimulates the Na^+-K^+-ATPase, a resulting drop in internal Na^+ may cause stimulation of the forward mode of NCX, producing depolarization (Kim *et al.*, 2005). Under these conditions, ouabain is expected to inhibit partially K^+-induced depolarization. However, as shown in Figure 6, additional administration of 500 μM ouabain to a K^+-enriched solution further depolarized endothelium by 7.2 ± 0.9 mV ($n=5$), providing no evidence that rise in external K^+ stimulates the Na^+-K^+-ATPase in rat aortic endothelium.

ACh-induced, endothelium-derived NO was demonstrated to promote a sustained hyperpolarization of smooth muscle cells in rat aorta (Vanheel *et al.*, 1994). This effect of NO may be mediated by the opening of K^+ channels (Satake *et al.*, 1997) or by the stimulation of the smooth muscle Na^+-K^+-ATPase (Rapoport *et al.*, 1985). To rule out the possibility that NO-attributed smooth muscle hyperpolarization is transmitted to the endothelium, thus promoting endothelial hyperpolarization to ACh, we examined the effect of the NO synthase inhibitor N^G-monomethyl-L-arginine monoacetate (L-NMMA) (200 μM) on ACh-evoked endothelial hyperpolarization. If a sustained smooth muscle cell hyperpolarization stimulated by endothelium-derived NO is transmitted to endothelial cells, inhibition of NO synthesis would partially inhibit the sustained component of endothelial hyperpolarization evoked by ACh. However, the hyperpolarization was unaffected by L-NMMA ($n=4$) (Figure 7). Further administration of 500 μM ouabain in the continued presence of L-NMMA attenuated the sustained hyperpolarization to ACh (at 5 min, from 16.0 ± 0.7 mV, $n=3$ to 7.9 ± 1.8, $n=3$) (data not shown).

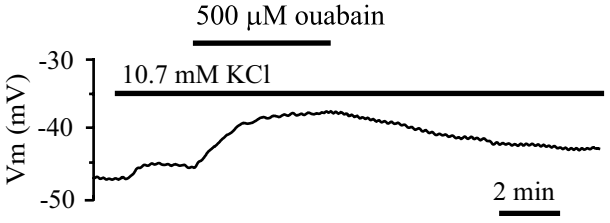

Figure 6 Original tracing of the membrane potential responses to increased KCl (from 4.7 to 10.7 mM) followed by 500 μM ouabain administration.

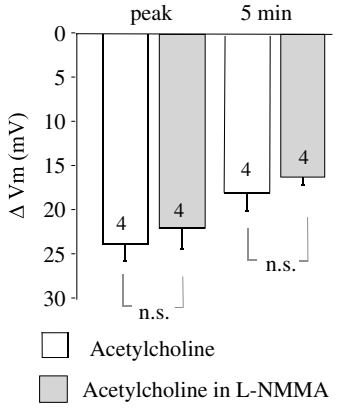

Figure 7 Effect of NO synthase inhibitor L-NMMA (200 μM) on the mean changes in membrane potential of endothelial cells evoked by 2 μM acetylcholine. The changes in membrane potential were calculated as the difference between the membrane potential values before and after acetylcholine administration. Numbers associated with bar plot indicate the number of aorta preparations.

Discussion

In the present study, the role of Na^+-K^+-ATPase in the regulation of endothelial membrane potential at rest, during K^+ reintroduction, and following ACh administration was evaluated in rat aorta, *in vitro*. Inhibition of the Na^+-K^+-ATPase by either withdrawal of external K^+ or with 500 μM ouabain produced a gradual endothelial cell depolarization of $\approx$11 mV, indicating strong basal activity of the Na^+-K^+-ATPase in endothelial cells. Activation of the Na^+-K^+-ATPase by reintroduction of external K^+ produced an abrupt endothelial hyperpolarization, which was abolished by 500 μM ouabain, confirming that this concentration of ouabain was effective at inhibiting Na^+-K^+-ATPase activity in rat aortic endothelium. In contrast, 500 nM ouabain failed to affect either the resting membrane potential or the hyperpolarization produced by K^+ reintroduction. Because of the different ouabain affinity among rodent Na^+-K^+-ATPase isozymes (Blanco and Mercer, 1998), the results obtained indicate that the ouabain-resistant α1 isoform, but not α2 or α3, is involved in setting the resting membrane potential in rat aortic endothelium. This conclusion is compatible with previous observations made in porcine aortic (Gruwel *et al*., 1995) and human umbilical vein (Oike *et al*., 1993) endothelial cells, which require high ouabain concentrations to inhibit the Na^+-K^+-ATPase. These results are also in agreement with the findings that the endothelium of large vessels predominantly expresses the α1 isoform of Na^+-K^+-ATPase (Zahler *et al*., 1996).

Inhibition of the Na^+-K^+-ATPase is expected to result in internal Na^+ accumulation and stimulation of the reversed mode of NCX. The latter, being electrogenic, is likely to attenuate the depolarizing effect caused by Na^+-K^+-ATPase inhibition. A lack of effect of benzamil on endothelial membrane potential in K^+-free solution provides no evidence for stimulation of the reversed NCX following Na^+-K^+-ATPase inhibition in rat aortic endothelial cells under the current experimental conditions. Similar conclusions have been drawn from experiments on cultured endothelial cells derived from rat aorta (Dong *et al*., 2004), bovine atria (Laskey *et al*., 1990) and pulmonary artery (Sage *et al*., 1991), human umbilical vein (Oike *et al*., 1993) and rat brain (Domotor *et al*., 1999), where Na^+-K^+-ATPase inhibition was without effect or decreased resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). These results are at variance with those obtained in intact rabbit cardiac valve endothelial cells (Li and van Breemen, 1995), where fura 2 fluorescent ratio was moderately increased by ouabain, indicating an increase in $[Ca^{2+}]_i$. The reason for this discrepancy is not easy to explain, but it may reflect the level of expression of different Na^+-K^+-ATPase α isoforms. Both α2 and α3 isoforms have been shown to be ouabain-sensitive and colocalized with NCX in plasma membrane microdomains that overlie the endoplasmic reticulum. These isoforms regulate $[Na^+]_i$ in a restricted cytosolic space between the plasma membrane and endoplasmic reticulum, whereas the α1 isoform is uniformly distributed over the cell surface (Juhaszova and Blaustein, 1997) and responsible for the regulation of bulk $[Na^+]_i$. Owing to the close proximity of α2/α3 subunits to the NCX, their inhibition may lead to stimulation of reverse NCX, whereas in vascular beds, where α2/α3 isoforms of Na^+-K^+-ATPase are dormant or absent, this may not be the case.

Another explanation for the aforementioned discrepancy may arise from variations in the resting $[Ca^{2+}]_i$ values and the contribution of the Na^+-K^+-ATPase to the resting membrane potential. The ouabain-induced membrane depolarization to $\sim$−30 mV observed in the present study decreases the driving force for basal Ca^{2+} entry. But operation of NCX in reversed mode requires a permissive $[Ca^{2+}]_i$ (Blaustein and Lederer, 1999). In native endothelial cells from rat aorta, the resting $[Ca^{2+}]_i$ was reported to be 95 nM (Usachev *et al*., 1995). Therefore, an anticipated increase in $[Na^+]_i$ caused by Na^+-K^+-ATPase inhibition that is accompanied by drop in $[Ca^{2+}]_i$ below 90 nM may not result in stimulation of the reverse NCX.

To quantify the change in ACh-induced hyperpolarization elicited by Na^+-K^+-ATPase inhibition, the amplitude and the duration of the hyperpolarization were simultaneously considered. A lack of effect of ouabain on the peak hyperpolarization to ACh observed in the present study is in agreement with previous findings where ouabain was tested either alone (Chen and Cheung, 1992) or in combination with low Ba^{2+} concentrations (Edwards *et al*., 1998; White and Hiley, 2000; Jiang *et al*., 2005). A novel finding in the present study is that the sustained hyperpolarization to ACh was attenuated by Na^+-K^+-ATPase inhibition either by ouabain or K^+-free solution. This effect was clearly observed after 5 min of hyperpolarization in the ouabain experiments and after 7 min when K^+-free solution was used. In the latter case, the difference in amplitude was not seen at 5 min, because the peak hyperpolarization in K^+-free solution was increased due to an enhanced driving force for K^+. However, the inhibitory effect of K^+-free solution on the sustained hyperpolarization was still observed at 5 min, as the decline in amplitude at this point was larger than that observed in the control solution.

In contrast, a low ouabain concentration that selectively inhibits α2 and α3 isoforms of the Na^+-K^+-ATPase was not effective in modifying either the resting membrane poten-

tial, the hyperpolarization to K^+ reintroduction or the hyperpolarization to ACh, suggesting that the α1, but not the α2 or α3 isoforms, mediates prolongation of the hyperpolarization to ACh in rat aortic endothelial cells. The results obtained are thus compatible with a previously reported observation that relatively high concentrations of ouabain (300 μM) are required to inhibit an endothelium-derived increase in $86Rb^+$ efflux induced by ACh in the rat aorta (Bray and Quast, 1991). Furthermore, wire myograph studies confirmed that ouabain at up to 1 mM is required for maximal inhibition of the relaxation in response to muscarinic stimulation (Rapoport *et al*., 1985; Hirano *et al*., 1992; Lee *et al*., 1992).

These results, however, are not in line with the observation that nanomolar concentrations of ouabain induce a bradykinin-induced $[Ca^{2+}]_i$ increase and NO release from rat aortic endothelial cells (Dong *et al*., 2004). This discrepancy may reflect differences in the mechanisms of action of ACh and bradykinin or the experimental conditions applied (transient application vs superfusion in the present study). Alternatively, the disparity may originate from possible alterations in $[Ca^{2+}]_i$ regulation in cultured vs *in situ* endothelial cells.

Although the precise mechanisms underlying prolongation of endothelial hyperpolarization to ACh by Na^+-K^+-ATPase remain to be determined, they may be connected either to internal Na^+ loading or a rise in extracellular K^+. Inhibition of NO synthesis by L-NMMA failed to affect endothelial hyperpolarization to ACh as well as prevent the inhibitory effect of ouabain. Therefore, it is unlikely that prolongation of the hyperpolarization is mediated by the stimulation of Na^+-K^+-ATPase of the smooth muscle cells by endothelium-derived NO with subsequent transmission of the signal to the endothelial cell layer.

The stimulant effect of K^+ on Na^+-K^+-ATPase has been proposed to underlie the EDHF-type response in rat hepatic and mesenteric arteries (Edwards *et al*., 1998). However, in a number of vascular beds, support for the involvement of this EDHF pathway has not been obtained (Quignard *et al*, 1999; Coleman *et al*., 2001). Based on the differences in ouabain affinity between rodent Na^+-K^+-ATPase isozymes (Blanco and Mercer, 1998), the present study indicates that the ouabain-resistant α1 isoform is implicated in the membrane potential modulation by ouabain in rat aortic endothelial cells. It is also known that α isoforms in rats differ by their affinities to extracellular K^+. A strong argument in support of our assumption that a rise in external K^+ does not mediate the increase in Na^+-K^+-ATPase activity in rat aortic endothelium is the finding that, whereas the α2 or α3 isoforms increase their activity in response to a small elevation in extracellular K^+, the α1 isoform is nearly fully activated at a physiological concentration of extracellular K^+ (Juhaszova and Blaustein, 1997; Blanco and Mercer, 1998; Weston *et al*., 2002). Indeed, in support of the view that the α1 isoform is predominantly responsible for the membrane potential modulation in response to ouabain in rat aortic endothelium, we demonstrated that an elevation in external K^+ produces endothelial cell depolarization; an effect that is not mediated by the forward mode of NCX secondary to stimulation of the Na^+-K^+-ATPase. Collectively, these observations do not favour the view that the rise in external K^+ stimulates the Na^+-K^+-ATPase in rat aortic endothelial cells.

Under normal physiological conditions, the Na pump is unsaturated with respect to intracellular Na^+ (Therien and Blostein, 2000), and therefore $[Na^+]_i$ is another potent determinant of pump activity. In endothelial cells from porcine aorta, a 14% increase in $[Na^+]_i$ produced by nystatin, augmented the Na pump rate approximately two-fold (Gruwel *et al*., 1995), indicating that small changes in $[Na^+]_i$ may have a substantial effect on the Na^+-K^+-ATPase activity. In the present study, ACh and ionomycin produced different patterns of hyperpolarization, indicating that the response to ACh is more complex and cannot simply be mimicked by an overall elevation of $[Ca^{2+}]_i$ in endothelial cells and probably reflects endoplasmic reticulum Ca^{2+} refilling. Previously it has been shown (Bondarenko, 2004) that NCX blockers and, importantly, external Na^+ withdrawal during the plateau phase of the hyperpolarization substantially inhibit the sustained endothelial hyperpolarization in response to ACh in rat aorta, suggesting that Na^+ influx activates the reversed NCX and possibly other Na^+ extrusion mechanisms following stimulation by ACh in the endothelial cells. This hypothesis is supported by results obtained with regard to several Na^+ entry pathways described in endothelial cells: various nonselective cationic channels including store-operated channels (Nilius and Droogmans, 2001) and the sodium–hydrogen exchanger (Fleming *et al*., 1994). Moreover, recent data show that endothelial cells from rat and human arteries are equipped with functional nicotinic ACh receptors that are Na^+ permeant (Macklin *et al*., 1998; Bruggmann *et al*., 2002). Collectively, these data support the view that intracellular Na^+ loading may underpin Na^+-K^+-ATPase stimulation by ACh in rat aortic endothelium.

In conclusion, the results obtained demonstrate that Na^+-K^+-ATPase inhibition either by ouabain or withdrawal of external K^+ attenuates the sustained component of endothelial hyperpolarization to ACh in rat aorta. These data indicate that Na^+-K^+-ATPase stimulation is at least partially involved in the ACh-induced hyperpolarization of endothelial cells. The precise mechanisms underlying this observation cannot be deducted from the present study, but may involve Na^+ loading into endothelial cells. Because a maintained hyperpolarization of endothelial cells is of primary importance in the smooth muscle hyperpolarization and relaxation, the results obtained suggest that suppression of endothelium-dependent relaxation by inhibitors of the Na^+-K^+-ATPase may be attained not only by their action on the smooth muscle Na^+-K^+-ATPase, but also on the endothelial Na^+-K^+-ATPase. Therefore, the physiological role of the Na^+-K^+-ATPase in endothelial cells may not only encompass a maintenance of electrochemical gradient for Na^+ and K^+ and contribution towards the resting membrane potential, but also a fine tuning of the electrochemical gradient for Ca^{2+} during stimulation with ACh.

Conflict of interest

The authors state no conflict of interest.

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