British Journal of Pharmacology (2008) 154, 982–990

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Endothelial nitric oxide attenuates Na^+/Ca^{2+} exchanger-mediated vasoconstriction in rat aorta

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Background and purpose: The Na^+/Ca^{2+} exchanger (NCX) may be an important modulator of Ca^{2+} entry and exit. The present study investigated whether NCX was affected by prostacyclin and nitric oxide (NO) released from the vascular endothelium, as NCX contains phosphorylation sites for PKA and PKG.
Experimental approach: Rat aortic rings were set up in organ baths. Tension was measured across the ring with a force transducer.
Key results: Lowering extracellular [Na+] ($[Na+]_o$) to 1.18 mM induced vasoconstriction in rat endothelium-denuded aortic rings. This effect was blocked by the NCX inhibitor KB-R7943 (2-2-[4-(4-nitrobenzyloxy)phenyl] ethyl isothiourea methanesulphonate; 1 μM). In endothelium-intact aortic rings, decreasing $[Na+]_o$ did not constrict the aortic rings significantly, but after treatment with the guanylate cyclase inhibitor ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; 1 μM) or the NOS inhibitor L-NAME (N^{ω}-nitro-L-arginine methyl ester; 50 μM), a vasoconstriction that was similar in size to that in endothelium-denuded preparations was evident. The vasorelaxation induced by the NO donor sodium nitroprusside sodium nitroprusside dihydrate (30 nM) was the same in the endothelium-denuded aortic rings preconstricted with either low Na^+ (1.18 mM), the thromboxane A2 agonist U46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; 0.1 μM) or high K^+ (80 mM).
Conclusions and implications: The results suggest that the endothelium inhibits NCX operation via guanylate cyclase/NO. This is stronger than for other constrictors such as phenylephrine and may relate to concomitant NCX-stimulated NO release from the endothelium. This finding may be important where NCX operates in reverse mode, such as during ischaemia, and highlights a new mechanism whereby the endothelium modulates Ca^{2+} homoeostasis in vascular smooth muscle.
British Journal of Pharmacology (2008) **154,** 982–990; doi:10.1038/bjp.2008.178; published online 12 May 2008

Keywords: Na^+/Ca^{2+} exchanger; aorta; nitric oxide; endothelium

Abbreviations: NCX, Na^+/Ca^{2+} exchanger; $[Na^+]_o$, extracellular Na^+ concentration

Introduction

The Na^+/Ca^{2+} exchanger (NCX) is an integral membrane protein responsible for the counter-transport of Na^+ and Ca^{2+} across the plasma membrane of virtually all animal cells. Three genes belonging to NCX family that code for NCX have been identified in mammals, namely NCX1 (Nicoll *et al*., 1990), NCX2 (Li *et al*., 1994) and NCX3 (Nicoll *et al*., 1996). NCX1 is widely expressed in diverse tissues including the heart, brain, skeletal muscle, smooth muscle, kidney, spleen and lung. The cloned cardiac NCX1 has been elucidated in rat aorta (Nakasaki *et al*., 1993). The present study focuses on NCX1 in rat aorta.

The energy and direction for net Ca^{2+} movement across NCX depends on the electrochemical gradient of Na^+, Ca^{2+} and K^+, the membrane potential and the transport stoichiometry (for review, see Philipson, 2002). NCX is only partially active at the normal resting $[Ca^{2+}]_i$ (~100 nM) in most cells; micromolar $[Ca^{2+}]_i$ above resting level is required for NCX to function fully. The exchanger is fully activated when intracellular $[Ca^{2+}]$ is in the low micromolar range (that is, at about the $[Ca^{2+}]_i$ expected during peak activity in many types of excitable and secretory cells). The intracellular Ca^{2+}-dependent activation might be a protective mechanism that only allows NCX to be turned on when $[Ca^{2+}]_i$ is high and off when $[Ca^{2+}]_i$ is low (DiPolo and Beauge, 2002).

The primary function of NCX is to remove the Ca^{2+} out of the cells (operating in forward mode), although it also mediates Ca^{2+} influx (operating in reverse mode) in some circumstances, for example, when intracellular Na^+ is increased or extracellular Na^+ decreased (Linck *et al*., 1998). These conditions occur during ischaemia due to overstimulation of excitatory receptors promoting Na^+ influx or the shutdown of the Na^+K^+ ATPase pump

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Received 13 March 2008; accepted 28 March 2008; published online 12 May 2008

preventing Na^+ extrusion, and reverse mode operations for NCX have been described for both neural and cardiac tissue (see Jeffs *et al*., 2007; Iwamoto *et al*., 2007). Reverse mode can be simulated experimentally by reducing extracellular Na^+.

Na^+/Ca^{2+} exchanger may also be modulated through PKs. Thus, Yamanaka *et al*. (2003) showed that the cAMP/PKA system stimulated NCX activity in porcine coronary artery smooth muscle cells. This is consistent with the upregulatory effect of cAMP/PKA system on NCX in rat neurons (He *et al*., 1998). However, both PKA and PKG have been shown to inhibit the activity of NCX in human cultured mesangial cells (Mene *et al*., 1993). Hence, the modulation of NCX by cyclic nucleotides clearly depends on the tissue and the experimental conditions. Little is known about the role of NCX in vascular smooth muscle, and this may be important given that both nitric oxide (NO) and prostacyclin activate PKG and PKA, respectively, and are important modulators of vascular function (see Mombouli and Vanhoutte, 1999).

The release of NO from the vascular endothelium activates the guanylate cyclase/cyclic GMP/PKG system in the smooth muscle and causes vasorelaxation. A number of mechanisms for this relaxation have been proposed and include inhibition of Ca^{2+} entry through L-type Ca^{2+} channels, facilitation of Ca^{2+} removal via Ca^{2+}-ATPase or uptake into intracellular stores, and decreased sensitivity of the contractile apparatus to Ca^{2+} by the activation of myosin light chain phosphatase (Sauzeau *et al*., 2000; Chitaley and Webb, 2002; Nishimura, 2006; see also review by Hirano, 2007). However, the potential for endothelial NO to modulate NCX has not been elucidated and this could be important given the emerging role of NCX in Ca^{2+} homoeostasis. Also, endothelial-derived prostacyclin has been shown to inhibit similar Ca^{2+} signalling events to cause vasorelaxation through an effect on the cyclic AMP/PKA pathways (see Mombouli and Vanhoutte, 1999).

Hence, the aim of the present study was to determine whether the operation of the NCX could be modulated by the endothelium and, if so, whether the NO and prostacyclin pathways are involved. The model used was that of a rat aorta, which has been shown previously to contain NCX and to display vasoconstriction in response to low extracellular Na^+, owing to NCX operating in reverse mode.

Methods

Tissue preparation for functional studies

Male Sprague–Dawley rats (5- to 7-weeks old, weighing 200–350 g, 87 rats in total) were anaesthetized with thiopentone sodium (100 mg kg−1 i.p.) and then killed by decapitation. The thoracic aorta was removed, placed in a cold physiological salt solution (PSSA), cleaned of surrounding fat and connective tissue and cut into equal-sized ring segments (4 mm in length). In some cases, the endothelium was removed by gentle rubbing of the luminal surface with a plastic rod. Each ring was suspended in an organ bath containing 2 mL of PSSA. Tissues were washed thoroughly by replacing the PSS repeatedly and were then allowed to equilibrate for a period of 60 min under 2 g of resting tension. Tissues that failed to produce a 0.5 g increase in tension to phenylephrine (1 μM) were rejected. The successful removal of endothelial cells was confirmed by the inability of ACh (1 μM) to induce relaxation in the presence of phenylephrine.

For the study of NCX operating in reverse mode, the extracellular $[Na^+]$ was reduced from 144.18 to 1.18 mM. Low Na^+ PSS (PSSB) was obtained by substituting choline chloride for NaCl, and was bubbled with 100% O_2 and the pH adjusted to 7.4 with KOH. In the control group, the normal PSS (PSSA) was bubbled with 100% O_2 and the pH was adjusted to 7.4 with NaOH (Horiguchi *et al*., 2001).

Composition of PSS

Normal physiological salt solution (PSSA) (in mM): NaCl 143; KCl 4.7;. NaH_2PO_4 1.18; $MgSO_4 \cdot 7H_2O$ 1.17; $CaCl_2 \cdot 2H_2O$ 2.5; glucose 11 and HEPES 5.

Low Na^+ physiological salt solution (PSSB) (in mM): choline chloride 143; KCl 4.7; NaH_2PO_4 1.18; $MgSO_4 \cdot 7H_2O$ 1.17; $CaCl_2 \cdot 2H_2O$ 2.5; glucose 11; and HEPES 5.

Drugs and materials

The following drugs were used: L-phenylephrine hydrochloride, ACh chloride, 9-(2-tetrahydrofuryl) adenine (SQ 22536), indomethacin, guanethidine monosulphate, sodium nitroprusside dihydrate (SNP), N^{ω}-nitro-L-arginine methyl ester (L-NAME) and thiopentone sodium from Sigma (St Louis, USA), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) from Cayman Chemical (MI, USA), KB-R7943 (2-2-[4-(4-nitrobenzyloxy)phenyl] ethyl isothiourea methanesulphonate) from TOCRIS (Ellisville, MI, USA) and 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619) from BIOMOL (Plymouth Meeting, PA, USA). Phenylephrine and ACh were dissolved in distilled water, and the remaining drugs were dissolved in dimethyl sulfoxide. The final concentration of vehicle was no more than 0.1% (v/v). Buffer salts and chemicals were obtained from Sigma (St Louis, MO, USA) unless otherwise stated.

Drug/molecular target nomenclature conforms with the *British Journal of Pharmacology Guide to Receptors and Channels* (Alexander *et al*., 2007).

Data analysis

Results are expressed as mean ± s.e.mean and were considered different when $P<0.05$. Multiple comparisons were determined using ANOVA in GbStat package (version 7.0, Dynamic Microsystems Inc., Silver Spring, MD, USA).

Results

Constriction in rat aortic rings induced by low Na^+

In rat endothelium-denuded aortic rings, lowering $[Na^+]_o$ to 1.18 mM induced immediate constriction and phase vasomotion (Figures 1a and b). The adrenergic neuron blocker guanethidine (3 μM), which was present for 30 min before

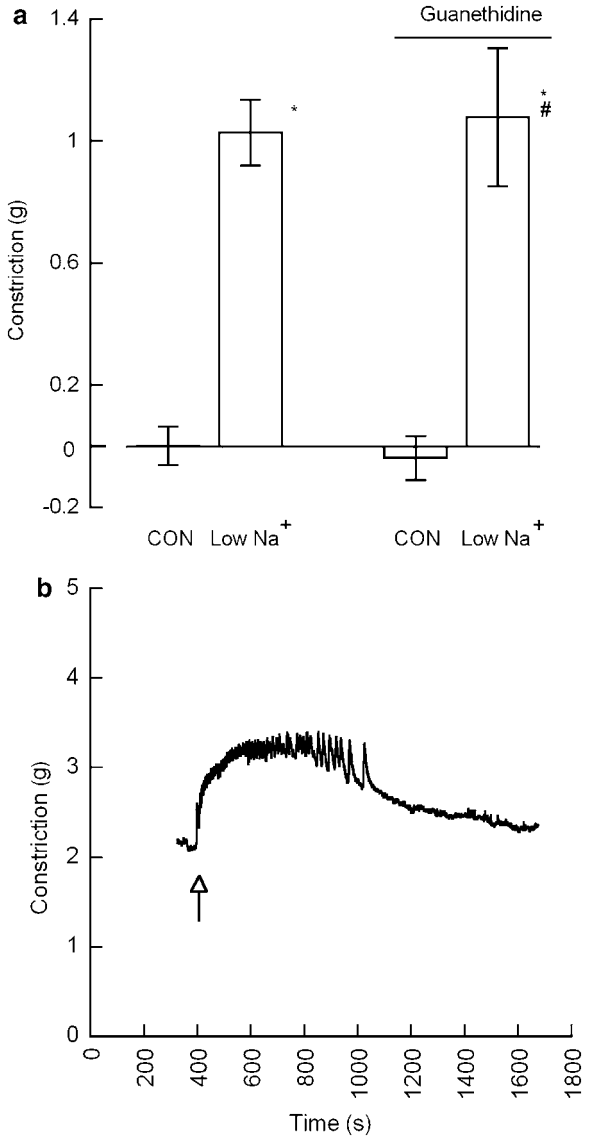

Figure 1 The effect of low Na^+ in rat endothelium-denuded aortic rings. Rat aortic rings were bathed in normal physiological salt solution (PSS) (144.18 mM Na^+, CON). This was replaced with low Na^+ PSS (1.18 mM, low Na^+). (**a**) Constriction after low Na^+ in the absence and presence of guanethidine (3 μM); means ± s.e.mean are shown and $n=5$–7 for each group. The *y* axis represents the response in grams (g). *Significant difference from the respective control (CON) $P<0.05$, Student's *t*-test. #Guanethidine had no significant effect on the low Na^+-induced constriction, $P>0.05$, two-way ANOVA. (**b**) An original trace of the effect of low Na^+.

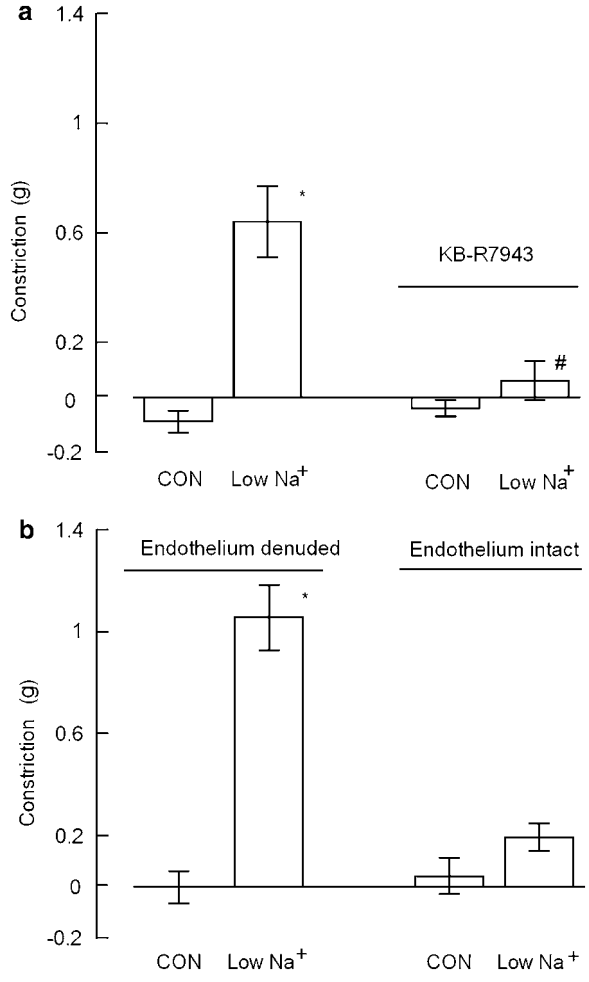

Figure 2 Effect of KB-R7943 (1 μM) in endothelium-denuded and intact aortic rings. Rat aortic rings were bathed in normal physiological salt solution (PSS) (144.18 mM Na^+, CON). This was replaced with low Na^+ PSS (1.18 mM, low Na^+). (**a**) Constrictor responses after the addition of low Na^+ in the absence and presence of KB-R7943 (1 μM). (**b**) The effect of low Na^+ in endothelium-denuded and intact aortic rings. The columns represent means ± s.e.mean, $n=5$–7 for each group. The *y* axis represents the response in grams (g). *Significant difference from the respective control (CON) $P<0.05$, Student's *t*-test. #Significant inhibition by KB-R7943 (2-2-[4-(4-nitrobenzyloxy)phenyl] ethyl isothiourea methanesulphonate; 1 μM), $P<0.05$, two-way ANOVA.

testing, showed no effect on low Na^+-induced constriction in these endothelium-denuded aortic rings (Figure 1a), indicating that sympathetic nerves were not involved. Guanethidine was found to be an effective adrenergic neuron transmission blocker in rat aorta at a concentration of 3 μM (Berry *et al*., 1992; Toma *et al*., 1995). The NCX inhibitor KB-R7943 (1 μM) significantly attenuated this effect of lowering $[Na^+]_o$ (Figure 2a). In endothelium-intact vessels, lowering $[Na^+]_o$ did not constrict the aortic rings significantly (Figure 2b).

The effect of ODQ on constriction in rat aortic rings induced by low Na^+

The soluble guanylate cyclase inhibitor ODQ was used to determine whether NO-linked processes might be involved in the modulation of NCX. In endothelium-intact aortic rings, ODQ (1 μM) greatly amplified the vasoconstrictor response to lowering $[Na^+]_o$ but had no effect on the low Na^+-induced constriction when the endothelium was removed (Figure 3a).

The effect of L-NAME on constriction in rat aortic rings induced by low Na^+

The NOS inhibitor L-NAME was also used to determine whether NO-linked processes might be involved in the

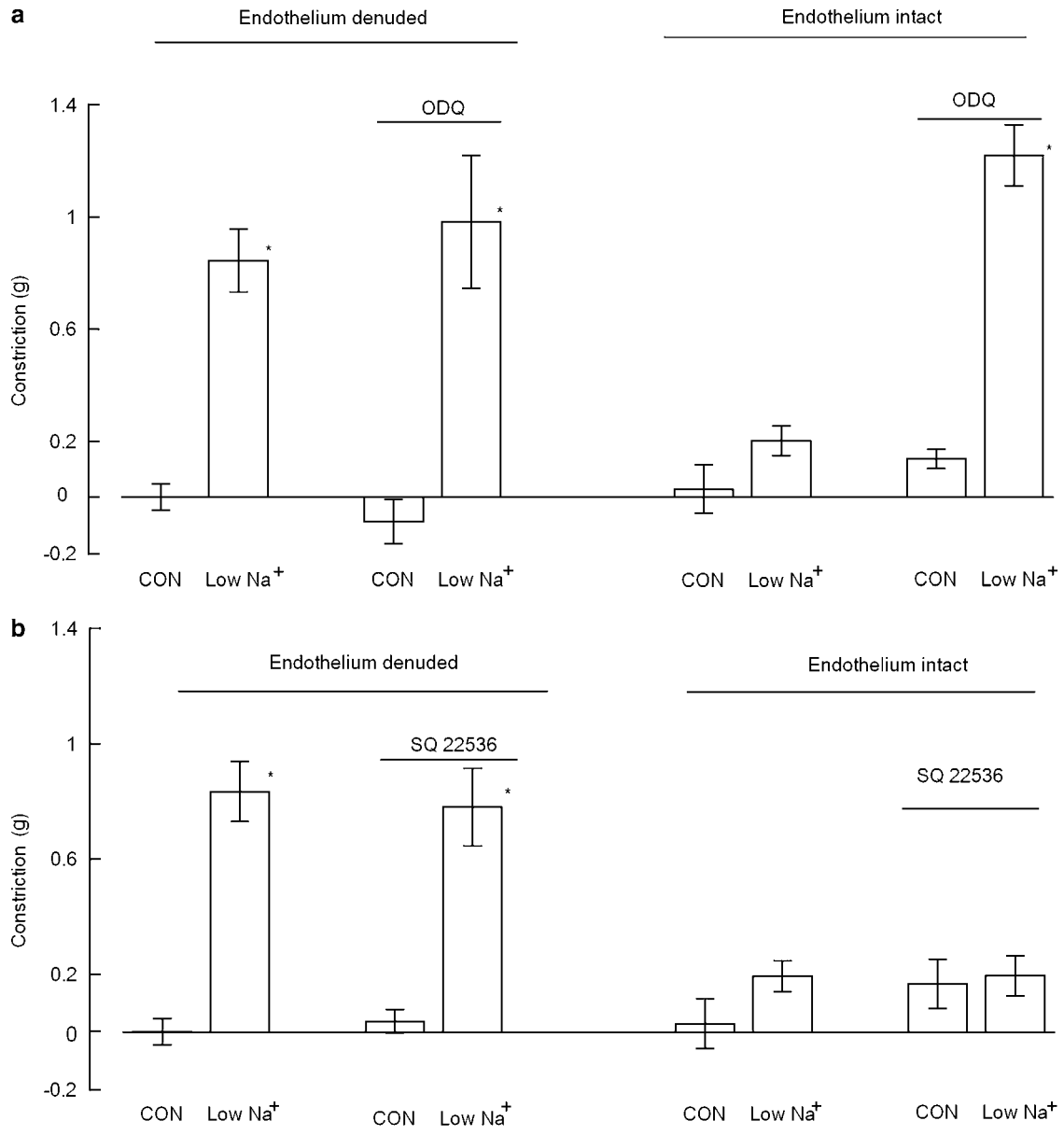

Figure 3 Effect of 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 9-(2-tetrahydrofuryl) adenine (SQ 22536) on constriction induced by lowering $[Na^+]_o$ in endothelium-denuded and intact aortic rings. Rat aortic rings were bathed in normal PSS (144.18 mM Na^+, CON). This was replaced with low Na^+ physiological salt solution (PSS) (1.18 mM, low Na^+). (**a**) Constrictor responses after the addition of low Na^+ in the absence and presence of ODQ (1 μM). (**b**) Constrictor responses after the addition of low Na^+ in the absence and presence of SQ 22536 (100 μM). The columns represent means ± s.e.mean, $n = 5–7$ for each group. The *y* axis represents the response in grams (g). *Significant difference from the respective control (CON) $P < 0.05$, Student's *t*-test.

endothelial modulation of NCX. In endothelium-intact aortic rings, L-NAME (50 μM) greatly amplified the vasoconstriction induced by lowering extracellular Na^+ but had no effect in endothelium-denuded aortic rings (Figure 4a).

The effect of SQ 22536 on constriction in rat aortic rings induced by low Na^+

The involvement of the cAMP pathway was investigated. In both endothelium-denuded and intact vessels, the aortic rings were incubated with the adenylyl cyclase blocker SQ 22536. SQ 22536 (100 μM) showed no significant effect on the low Na^+-induced vasoconstriction in either endothelium-denuded or intact aortic rings (Figure 3b).

The effect of indomethacin on constriction in rat aortic rings induced by low Na^+

To ascertain if there is any alternative pathway of endothelial modulation of NCX apart from NO, the production of prostacyclin was inhibited using the COX inhibitor indomethacin. Indomethacin (10 μM) had no effect on low Na^+-induced vasoconstriction in endothelium-intact aortic rings (Figure 4b).

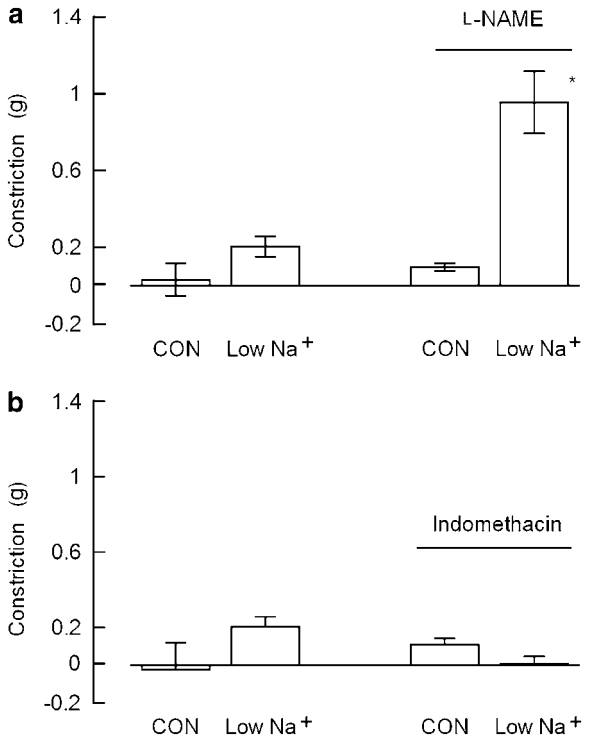

Figure 4 Effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and indomethacin on constriction induced by lowering $[Na^+]_o$ in endothelium-intact aortic rings. Rat aortic rings were bathed in normal physiological salt solution (PSS) (144.18 mM Na^+, CON). This was replaced with low Na^+ PSS (1.18 mM, low Na^+). (**a**) Constrictor responses after the addition of low Na^+ in the absence and presence of L-NAME (50 μM). (**b**) Constrictor responses after the addition of low Na^+ in the absence and presence of indomethacin (10 μM). The columns represent mean ± s.e.mean, $n = 5$–9 for each group. The *y* axis represent the response in grams (g). *Significant difference from the respective control (CON) $P < 0.05$, Student's *t*-test.

Table 1 The maximum constriction induced by low Na^+ or U46619 in rat aortic rings

Name of vasoconstrictor	*Constriction (g)*
Low Na^+ (1.18 mM)	1.45 ± 0.23
U46619 (0.1 μM)	1.30 ± 0.22

Abbreviation: U46619, 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$.

The effect of SNP in rat aortic rings after preconstriction with low Na^+ or U46619

In an attempt to obtain direct evidence for the involvement of NO in the operation of NCX, the NO donor SNP was studied. Endothelium-denuded aortic rings were preconstricted to the same extent by different means: low Na^+ (1.18 mM), or the thromboxane A_2 agonist U46619 (0.1 μM). The absolute values of maximum constriction are shown in Table 1. SNP (30 nM) was added after each of these treatments and produced a vasorelaxation. The vasorelaxation to SNP after preconstriction with low Na^+ was not significantly different from that after preconstriction with U46619 (Figure 5a).

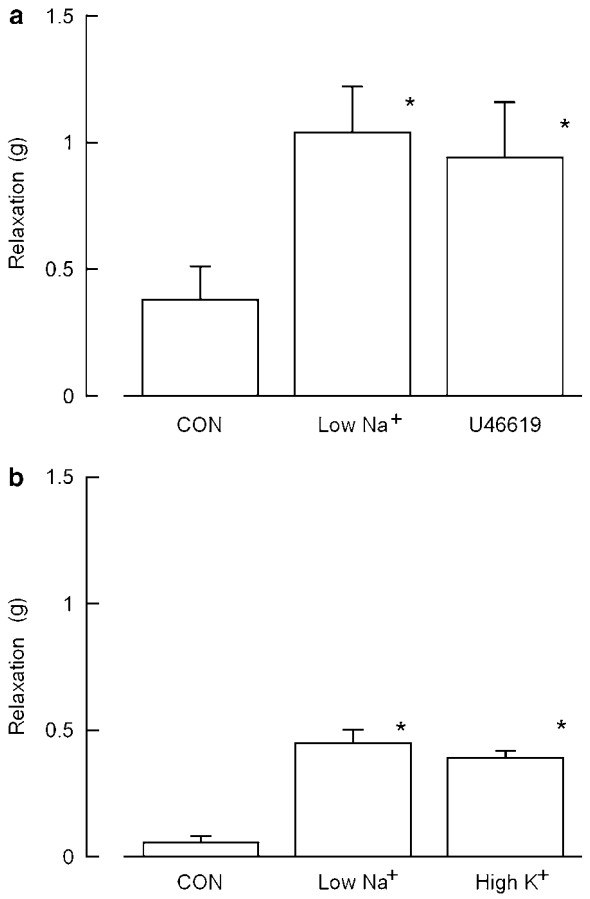

Figure 5 Effect of sodium nitroprusside dihydrate (SNP) (30 nM) in endothelium-denuded aortic rings preconstricted by different means. Rat aortic rings were bathed in normal physiological salt solution (PSS) (144.18 mM Na^+) and preconstricted by different means. SNP (30 nM) was added after the constriction had reached a plateau. (**a**) SNP induced vasorelaxation after preconstriction by either low Na^+ (1.18 mM) or U46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$; 0.1 μM); CON (0.1% DMSO). (**b**) SNP induced vasorelaxation after preconstriction by either low Na^+ (1.18 mM) or high K^+ (80 mM); CON (0.1% ethanol). The columns represent mean ± s.e.mean, $n = 5$–6 for each group. *Significant difference from the respective time control (CON) $P < 0.05$, Student's *t*-test. DMSO, dimethyl sulfoxide.

Table 2 The maximum constriction induced by low Na^+ or high K^+ in rat aortic rings

Name of vasoconstrictor	*Constriction (g)*
Low Na^+ (1.18 mM)	0.83 ± 0.12
High K^+ (80 mM)	1.09 ± 0.10

The effect of SNP in rat aortic rings after preconstriction with low Na^+ or high K^+

In another series of experiments, endothelium-denuded aortic rings were preconstricted to the same extent with either low Na^+ (1.18 mM) or high K^+ (80 mM). The absolute values of maximum constriction are shown in Table 2. SNP (30 nM) was added after each of these treatments and produced a vasorelaxation. This concentration of SNP was used, as it was on the slope of the concentration–response

curve for SNP. The vasorelaxation to SNP after preconstriction with low Na^+ was not significantly different from that after preconstriction with high K^+ (Figure 5b).

Discussion

In the present study, the role of the vascular endothelium in modulating vasoconstriction mediated through the NCX was investigated. In endothelium-denuded aortae, lowering extracellular [Na^+] (144.18–1.18 mM) induced an immediate constriction. Other studies have also shown a constriction induced by lowering Na^+ in vascular tissue (Reuter *et al*., 1973; Ashida and Blaustein, 1987; Bova *et al*., 1988; Maseki *et al*., 1990; Kim *et al*., 1999; Horiguchi *et al*., 2001; Rebolledo *et al*., 2006). The constriction is most likely due to the inflow of Ca^{2+} through NCX, as reducing the Na^+ gradient across the membrane makes the exchanger operate in reverse mode (Horiguchi *et al*., 2001; Schweda *et al*., 2001; Takai *et al*., 2004). Indeed, in the present study, the constriction was blocked by the NCX inhibitor KB-R7943 (1 μM). Other studies have also shown that low Na^+-induced vasoconstriction is blocked by NCX inhibitors (Kim *et al*., 1999; Schweda *et al*., 2001; Takai *et al*., 2004; Rebolledo *et al*., 2006). It should be noted that we used KB-R7943 at 1 μM, as higher concentrations have been shown to have nonspecific effects (Watano *et al*., 1996; Pintado *et al*., 2000; Matsuda *et al*., 2001; Reuter *et al*., 2002; Tanaka *et al*., 2002).

As NCX is also present in neurons, other workers have suggested alternate mechanisms to explain low Na^+-induced constriction, such as the stimulation of sympathetic nerve endings in rabbit aorta (Karaki and Urakawa, 1977). However, in the present study, this possibility was excluded, as the adrenergic neuron blocking drug guanethidine had no effect on low Na^+-induced constriction in rat aorta. This agrees with the observations of Ashida and Blaustein (1987) that low Na^+-induced vasoconstriction was maintained in the presence of the α-adrenoceptor blocker phentolamine in rat aorta.

A key question is: What role does the vascular endothelium play in this low Na^+-induced constriction? It is well established that the vascular endothelium, by releasing NO, has an important buffering effect on vasoconstrictor agents (Bredt and Snyder, 1994; Gryglewski, 2005). In the present study in rat aortic rings, in endothelium-intact vessels, lowering $[Na^+]_o$ did not constrict the aortic rings significantly, which was in contrast to the marked constriction seen when the endothelium was removed. To the best of our knowledge, this is the first time Na^+ removal has been shown to induce a different vasoconstrictor effect in endothelium-denuded vessels from that in intact vessels.

The vascular endothelium exerts its regulatory effect on vessel contractility through the release of a variety of substances. One of these is prostacyclin (Bredt and Snyder, 1994; Denninger and Marletta, 1999). Prostacyclin activates G-protein linked prostacyclin receptors (Wedel and Garbers, 1997) on vascular smooth muscle, and these receptors stimulate adenylyl cyclase to produce cAMP, which in turn activates PKA (McAdam *et al*., 1999; Cheng *et al*., 2002). Sites have been identified (on the intracellular loop) for PKA phosphorylation of NCX, but there are conflicting results regarding the modulation of NCX by the cAMP/PKA pathway; both upregulation (He *et al*., 1998; Perchenet *et al*., 2000; Yamanaka *et al*., 2003) and downregulation (Mene *et al*., 1993) have been demonstrated. However, in the present study, the adenylyl cyclase inhibitor SQ 22536 did not affect the low Na^+-induced vasoconstriction in endothelium-intact aortae. At the concentration of 100 μM, SQ 22536 has been shown to be an effective inhibitor of adenylyl cyclase in rat aorta (Lo *et al*., 2005; Wu *et al*., 2005; Morello *et al*., 2006). Others have also shown that PKA has no effect on NCX operations in cardiac myocytes (Ginsburg and Bers, 2005; Lin *et al*., 2006). Furthermore, in the present study, blocking prostacyclin synthesis with the non-selective COX inhibitor indomethacin (Vane and Botting, 1987; Perez-Vizcaino *et al*., 1999) also had no effect on low Na^+-induced constriction in endothelium-intact aortae. At the concentration used (10 μM), indomethacin has been shown to be an effective inhibitor of COX in rat aorta (Rapoport *et al*., 2000; Sofola *et al*., 2003; Ashraf *et al*., 2004; Molin and Bendhack, 2004). These results rule out the involvement of prostacyclin in the endothelial modulation of low Na^+-induced vasoconstriction.

The most prominent endothelium-dependent vasodilator is NO, which is formed by NOS and released by the vascular endothelium to activate soluble guanylate cyclase in the vascular smooth muscle (Bredt and Snyder, 1994; Denninger and Marletta, 1999). The synthesis and release of NO from the endothelium is due to the activation of NOS, stimulated by an increase in intracellular calcium (Binko *et al*., 1998; Molin and Bendhack, 2004). In the present study, two sets of results confirmed NO inhibition of low Na^+-induced vasoconstriction when the endothelium was present. Firstly, ODQ, a soluble guanylate cyclase inhibitor (Shelkovnikov *et al*., 2004), potentiated the low Na^+-induced constriction in aortae with intact endothelium to a level similar to that observed in endothelium-denuded aortae. In the absence of endothelium, the low Na^+-induced constriction was not affected by ODQ. This indicates that soluble guanylate cyclase was involved in the response and suggests that NO release was involved. This was confirmed by the finding that the NOS inhibitor L-NAME also enhanced the low Na^+-induced constriction in aortae with intact endothelium. Both ODQ (Feelisch *et al*., 1999; Chalupsky *et al*., 2004; Shelkovnikov *et al*., 2004) and L-NAME (Feelisch *et al*., 1999; Yang *et al*., 1999) at the concentrations used in the present study have been shown to be effective in rat aorta. To our knowledge, this is the first time NO has been shown to inhibit vasoconstriction mediated by NCX.

In the present study, we could not demonstrate a low Na^+-induced constriction in the rat endothelium-intact aorta and wondered why this has not been observed in other studies. Previous studies fall into several categories: those using isolated smooth muscle cells (Karaki and Urakawa, 1977; Linck *et al*., 1998; Kim *et al*., 1999; Takai *et al*., 2004; Rebolledo *et al*., 2006), those using strips of vessels where endothelial damage is probable due to physical handling (Bova *et al*., 1988; Maseki *et al*., 1990). Indeed, differences in the physical handling of vessels were responsible for the initial discovery of endothelium-derived

relaxing factor (Furchgott and Zawadzki, 1980), now known to be NO. In some other studies, the endothelium was removed (Motley *et al*., 1993). In each of these categories, it would be unlikely that an inhibitory effect of the endothelium would be observed. However, in some studies, a substantial constriction to low Na^+ has been observed in the presence of an intact endothelium, and these all involve small resistance vessels, such as in the rat isolated perfused kidney (Schweda *et al*., 2001) and rat arterioles (Horiguchi *et al*., 2001) and in our own study in rat cremaster arterioles (Zhao *et al*., 2004). It is unclear why endothelial dampening was not as evident in these tissues, but it is noteworthy that NO forms only a component of the endothelial response in those tissues that have a substantial input from endothelium derived hyperpolarising factor (EDHF): rat arterioles (Hilgers and Webb, 2007) and rat perfused kidney (Vargas and Osuna, 1996).

In the present study, the role of the endothelium appears to be more prominent in the vasoconstriction mediated by low Na^+ constriction than in that mediated by other means, as in the presence of a functional endothelium hardly any constriction to low Na^+ was evident, whereas endothelium removal revealed a substantial effect. However, in a previous study, the α-adrenoceptor agonist phenylephrine was found to constrict rat aortic rings markedly in the presence of the endothelium, and endothelium removal had only a small enhancing effect on the vasoconstriction (van der Zypp *et al*., 2000). Similar results were seen with other vasoconstrictors, for example, U46619 (Hahnenkamp *et al*., 2004). This suggests that low Na^+ constriction is inhibited more in the presence of the endothelium than in other constrictor mechanisms. This could be due to NO/cyclic GMP modulation of NCX, as NCX is inhibited by PKG human mesangial cells (Mene *et al*., 1993). To test this hypothesis, the endothelium was removed and NO added to the system in the form of sodium nitroprusside; three different forms of preconstriction were used U46619, a thromboxane A2 mimetic, which operates through the phospholipase C/IP_3 signalling pathway (Streefkerk *et al*., 2002); high K^+ ,which depolarizes smooth muscle and opens voltage-dependent Ca^{2+} channels (O'Donnell and Wanstall, 1987; Mendizabal *et al*., 2000); and the low Na^+, which acts via the NCX mechanism. SNP did not produce a more significant vasorelaxation of the low Na^+-induced constriction compared with that induced by other agents. This suggests that the activity of the NCX is not uniquely sensitive and indicates that it is unlikely that the exceptional sensitivity of NCX to inhibition by the endothelium occurs at the level of the smooth muscle cell.

The most likely explanation for the exceptional inhibitory effect of the endothelium on low Na^+ constriction is that low Na^+ induces the release of NO from the endothelium. It is well established that there is a basal release of NO from the endothelium (Bredt and Snyder, 1994; Li and Forstermann, 2000; Maxwell, 2002), and this may be simply inhibiting the activity of NCX. Additionally, NCX has been shown to modulate calcium homoeostasis in vascular endothelial cells (Sedova and Blatter, 1999; Paltauf-Doburzynska *et al*., 2000; Moccia *et al*., 2002; Wang *et al*., 2002; Liang *et al*., 2004). Therefore, lowering Na^+ may release NO from the endothelium by inducing the inflow of Ca^{2+} through endothelial NCX. Indeed, NCX has been implicated in the production and release of NO in endothelial cells, such as rat aortic endothelial cells (Schneider *et al*., 2002; Ogura *et al*., 2004), porcine aortic endothelial cells (Teubl *et al*., 1999) and cardiac microvascular endothelial cells (Kaye and Kelly, 1999). Also, NO release due to intracellular Na^+ loading has been shown to be blocked by the NCX inhibitor KB-R7943 (Schneider *et al*., 2002). Thus, the attenuation of low Na^+ vasoconstriction induced by the endothelium, observed in the present study, may be due to a simultaneous enhancement of NO release from the endothelium.

In summary, the present results indicate that NCX-mediated vasoconstriction is regulated by the endothelium via guanylate cyclase/NO in the rat aorta. The underlying mechanisms probably involve the enhancement of NO release from the endothelium and do not involve a direct effect of NO on NCX in the smooth muscle, as the relaxation response to SNP in aortae constricted by the addition of low Na^+ was similar to that induced in preparations preconstricted by other agents. This finding may be important under pathological conditions where NCX operates in reverse mode, such as during ischaemia. In ischaemia, NO release from the endothelium is diminished (Schulz *et al*., 2004) and this would unmask the effects of NCX, which under ischaemic conditions operates in reverse mode and therefore mediates vasoconstriction (Wei *et al*., 2007). Hence, NCX may exacerbate overall vasoconstriction in ischaemic conditions.

Conflict of interest

The authors state no conflict of interest.

References

Alexander SPH, Mathie A, Peters JA (2007). Guide to receptors and channels (GRAC), 2nd edition (2007 revision). *Br J Pharmacol* **150** (Suppl 1): S1–S168.

Ashida T, Blaustein MP (1987). Regulation of cell calcium and contractility in mammalian arterial smooth muscle: the role of sodium–calcium exchange. *J Physiol* **392**: 617–635.

Ashraf MZ, Hussain ME, Fahim M (2004). Endothelium mediated vasorelaxant response of garlic in isolated rat aorta: role of nitric oxide. *J Ethnopharmacol* **90**: 5–9.

Berry JL, Small RC, Hughes SJ, Smith RD, Miller AJ, Hollingsworth M *et al*. (1992). Inhibition by adrenergic neurone blocking agents of the relaxation induced by BRL 38227 in vascular, intestinal and uterine smooth muscle. *Br J Pharmacol* **107**: 288–295.

Binko J, Murphy TV, Majewski H (1998). 17Beta-oestradiol enhances nitric oxide synthase activity in endothelium-denuded rat aorta. *Clin Exp Pharmacol Physiol* **25**: 120–127.

Bova S, Cargnelli G, Luciani S (1988). Na/Ca exchange and tension development in vascular smooth muscle: effect of amiloride. *Br J Pharmacol* **93**: 601–608.

Bredt DS, Snyder SH (1994). Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem* **63**: 175–195.

Chalupsky K, Lobysheva I, Nepveu F, Gadea I, Beranova P, Entlicher G *et al*. (2004). Relaxant effect of oxime derivatives in isolated rat aorta: role of nitric oxide (NO) formation in smooth muscle. *Biochem Pharmacol* **67**: 1203–1214.

Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T *et al*. (2002). Role of prostacyclin in the cardiovascular response to thromboxane A_2. *Science* **296**: 539–541.

Chitaley K, Webb RC (2002). Nitric oxide induces dilation of rat aorta via inhibition of rho-kinase signaling. *Hypertension* **39**: 438–442.

Denninger JW, Marletta MA (1999). Guanylate cyclase and the [ccirf]NO/cGMP signaling pathway. *Biochim Biophys Acta* **1411**: 334–350.

DiPolo R, Beauge L (2002). Ionic ligand interactions with the intracellular loop of the sodium–calcium exchanger. Modulation by ATP. *Prog Biophys Mol Biol* **80**: 43–67.

Feelisch M, Kotsonis P, Siebe J, Clement B, Schmidt HH (1999). The soluble guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3,-a] quinoxalin-1-one is a nonselective heme protein inhibitor of nitric oxide synthase and other cytochrome P-450 enzymes involved in nitric oxide donor bioactivation. *Mol Pharmacol* **56**: 243–253.

Furchgott RF, Zawadzki JV (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**: 373–376.

Ginsburg KS, Bers DM (2005). Isoproterenol does not enhance Ca-dependent Na/Ca exchange current in intact rabbit ventricular myocytes. *J Mol Cell Cardiol* **39**: 972–981.

Gryglewski RJ (2005). Pharmacology of vascular endothelium. Delivered on 27 June 2004 at the 29th FEBS Congress in Warsaw. *FEBS J* **272**: 2956–2967.

Hahnenkamp K, Nollet J, Strumper D, Halene T, Rathman P, Mortier E *et al*. (2004). Bupivacaine inhibits thromboxane A_2-induced vasoconstriction in rat thoracic aorta. *Anesth Analg* **99**: 97–102.

He S, Ruknudin A, Bambrick LL, Lederer WJ, Schulze DH (1998). Isoform-specific regulation of the Na^+/Ca^{2+} exchanger in rat astrocytes and neurons by PKA. *J Neurosci* **18**: 4833–4841.

Hilgers RHP, Webb RC (2007). Reduced expression of SK_{Ca} and IK_{Ca} channel proteins in rat small mesenteric arteries during angiotensin II-induced hypertension. *Am J Physiol* **292**: H2275–H2284.

Hirano K (2007). Current topics in the regulatory mechanism underlying the Ca^{2+} sensitization of the contractile apparatus in vascular smooth muscle. *J Pharmacol Sci* **104**: 109–115.

Horiguchi S, Watanabe J, Kato H, Baba S, Shinozaki T, Miura M *et al*. (2001). Contribution of Na^+/Ca^{2+} exchanger to the regulation of myogenic tone in isolated rat small arteries. *Acta Physiol Scand* **173**: 167–173.

Iwamoto T, Watanabi Y, Kita S, Blaustein MP (2007). The role of the Na^+/Ca^{2+} exchange inhibitors: a new class of calcium regulators. *Cardiovasc Hematol Disord Drug Targets* **7**: 188–198.

Jeffs GJ, Meloni BP, Bakker AJ, Knuckey NW (2007). The role of the Na^+/Ca^{2+} exchanger (NCX) in neurons following ischaemia. *J Clin Neurosci* **14**: 507–514.

Karaki H, Urakawa N (1977). Possible role of endogenous catecholamines in the contractions induced in rabbit aorta by ouabain, sodium depletion and potassium depletion. *Eur J Pharmacol* **43**: 65–72.

Kaye DM, Kelly RA (1999). Expression and regulation of the sodium–calcium exchanger in cardiac microvascular endothelial cells. *Clin Exp Pharmacol Physiol* **26**: 651–655.

Kim JK, Kim SJ, Kim YC, So I, Kim KW (1999). Influence of extracellular Na^+ removal on cytosolic Ca^{2+} concentration in smooth muscle cells of rabbit cerebral artery. *J Smooth Muscle Res* **35**: 135–145.

Li H, Forstermann U (2000). Nitric oxide in the pathogenesis of vascular disease. *J Pathol* **190**: 244–254.

Li Z, Matsuoka S, Hryshko L, Nicoll D, Bersohn M, Burke E *et al*. (1994). Cloning of the NCX2 isoform of the plasma membrane Na^+–Ca^{2+} exchanger. *J Biol Chem* **269**: 17434–17439.

Liang W, Buluc M, van Breemen C, Wang X (2004). Vectorial Ca^{2+} release via ryanodine receptors contributes to Ca^{2+} extrusion from freshly isolated rabbit aortic endothelial cells. *Cell Calcium* **36**: 431–443.

Lin X, Jo H, Sakakibara Y, Tambara K, Kim B, Komeda M *et al*. (2006) Beta-adrenergic stimulation does not activate Na^+/Ca^{2+} exchange current in guinea pig, mouse and rat ventricular myocytes. *Am J Physiol* **290**: C601–C608.

Linck B, Qiu Z, He Z, Tong Q, Hilgemann DW, Philipson KD (1998). Functional comparison of the three isoforms of the Na^+/Ca^{2+} exchanger (NCX1, NCX2, NCX3). *Am J Physiol Cell Physiol* **274**: C415–C423.

Lo YC, Tsou HH, Lin RJ, Wu DC, Wu BN, Lin YT *et al*. (2005). Endothelium-dependent and -independent vasorelaxation by a theophylline derivative MCPT: roles of cyclic nucleotides, potassium channel opening and phosphodiesterase inhibition. *Life Sci* **76**: 931–944.

Maseki T, Abe T, Tomita T (1990). Pharmacological properties of contraction caused by sodium removal in muscle strips isolated from canine coronary artery. *Eur J Pharmacol* **190**: 355–363.

Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M *et al*. (2001). SEA0400, a novel and selective inhibitor of the Na^+–Ca^{2+} exchanger, attenuates reperfusion injury in the *in vitro* and *in vivo* cerebral ischemic models. *J Pharmacol Exp Ther* **298**: 249–256.

Maxwell AJ (2002). Mechanisms of dysfunction of the nitric oxide pathway in vascular diseases. *Nitric Oxide* **6**: 101–124.

McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA (1999). Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci USA* **96**: 272–277.

Mendizabal VE, Poblete I, Lomniczi A, Rettori V, Huidobro-Toro JP, Adler-Graschinsky E (2000). Nitric oxide synthase-independent release of nitric oxide induced by KCl in the perfused mesenteric bed of the rat. *Eur J Pharmacol* **409**: 85–91.

Mene P, Pugliese F, Cinotti GA (1993). Cyclic nucleotides inhibit Na^+/Ca^{2+} exchange in cultured human mesangial cells. *Exp Nephrol* **1**: 245–252.

Moccia F, Berra-Romani R, Baruffi S, Spaggiari S, Signorelli S, Castelli L *et al*. (2002). Ca^{2+} uptake by the endoplasmic reticulum Ca^{2+}-ATPase in rat microvascular endothelial cells. *Biochem J* **364**: 235–244.

Molin JC, Bendhack LM (2004). Clonidine induces rat aorta relaxation by nitric oxide-dependent and -independent mechanisms. *Vascul Pharmacol* **42**: 1–6.

Mombouli JV, Vanhoutte PM (1999). Endothelial dysfunction: from physiology to therapy. *J Mol Cell Cardiol* **31**: 61–74.

Morello S, Vellecco V, Alfieri A, Mascolo N, Cicala C (2006). Vasorelaxant effect of the flavonoid galangin on isolated rat thoracic aorta. *Life Sci* **78**: 825–830.

Motley ED, Paul RJ, Matlib MA (1993). Role of Na^+-Ca^{2+} exchange in the regulation of vascular smooth muscle tension. *Am J Physiol* **264**: H1028–H1040.

Nakasaki Y, Iwamoto T, Hanada H, Imagawa T, Shigekawa M (1993). Cloning of the rat aortic smooth muscle Na^+/Ca^{2+} exchanger and tissue-specific expression of isoforms. *J Biochem (Tokyo)* **114**: 528–534.

Nicoll DA, Longoni S, Philipson KD (1990). Molecular cloning and functional expression of the cardiac sarcolemmal Na^+–Ca^{2+} exchanger. *Science* **250**: 562–565.

Nicoll DA, Quednau BD, Qui Z, Xia Y-R, Lusis AJ, Philipson KD (1996). Cloning of a third mammalian Na^+–Ca^{2+} exchanger, NCX3. *J Biol Chem* **271**: 24914–24921.

Nishimura J (2006). Topics on the Na^+/Ca^{2+} exchanger: involvement of Na^+/Ca^{2+} exchanger in the vasodilator-induced vasorelaxation. *J Pharmacol Sci* **102**: 27–31.

O'Donnell SR, Wanstall JC (1987). Choice and concentration of contractile agent influence responses of rat aorta to vascular relaxant drugs. *J Pharm Pharmacol* **39**: 848–850.

Ogura J, Mitamura M, Someya A, Shimamura K, Takayama H, Aimi N *et al*. (2004). Mesaconitine-induced relaxation in rat aorta: role of Na^+/Ca^{2+} exchangers in endothelial cells. *Eur J Pharmacol* **483**: 139–146.

Paltauf-Doburzynska J, Frieden M, Spitaler M, Graier WF (2000). Histamine-induced Ca^{2+} oscillations in a human endothelial cell line depend on transmembrane ion flux, ryanodine receptors and endoplasmic reticulum Ca^{2+}-ATPase. *J Physiol* **524** (Part 3): 701–713.

Perchenet L, Hinde AK, Patel KC, Hancox JC, Levi AJ (2000). Stimulation of Na/Ca exchange by the beta-adrenergic/protein kinase A pathway in guinea-pig ventricular myocytes at 37 degrees C. *Pflugers Arch* **439**: 822–828.

Perez-Vizcaino F, Cogolludo AL, Zaragoza-Arnaez F, Fajardo S, Ibarra M, Lopez-Lopez JG *et al*. (1999). Vasodilator effects of sodium nitroprusside, levcromakalim and their combination in isolated rat aorta. *Br J Pharmacol* **128**: 1419–1426.

Philipson KD (2002). Na(+)–Ca(2+) exchange: three new tools. *Circ Res* **90**: 118–119.

Pintado AJ, Herrero CJ, Garcia AG, Montiel C (2000). The novel Na(+)/Ca(2+) exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. *Br J Pharmacol* **130**: 1893–1902.

Rapoport RM, Williams SP, Campbell AK (2000). Phorbol ester-induced phosphoinositide hydrolysis in rat aorta: role of cyclooxygenase products. *Life Sci* **67**: 1815–1824.

Rebolledo A, Speroni F, Raingo J, Salemme SV, Tanzi F, Munin V *et al*. (2006). The Na^+/Ca^{2+} exchanger is active and working in the reverse mode in human umbilical artery smooth muscle cells. *Biochem Biophys Res Commun* **339**: 840–845.

Reuter H, Blaustein MP, Haeusler G (1973). Na–Ca exchange and tension development in arterial smooth muscle. *Philos Trans R Soc Lond B Biol Sci* **265**: 87–94.

Reuter H, Henderson SA, Han T, Matsuda T, Baba A, Ross RS *et al*. (2002). Knockout mice for pharmacological screening: testing the specificity of Na^+–Ca^{2+} exchange inhibitors. *Circ Res* **91**: 90–92.

Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM, Bertoglio J *et al*. (2000). Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca^{2+} sensitization of contraction in vascular smooth muscle. *J Biol Chem* **275**: 21722–21729.

Schneider JC, El Kebir D, Chereau C, Mercier JC, Dall'Ava-Santucci J, Dinh-Xuan AT (2002). Involvement of Na(+)/Ca(2+) exchanger in endothelial NO production and endothelium-dependent relaxation. *Am J Physiol Heart Circ Physiol* **283**: H837–H844.

Schulz R, Kelm M, Heusch G (2004). Nitric oxide in myocardial ischemia/reperfusion injury. *Cardiovasc Res* **61**: 402–413.

Schweda F, Seebauer H, Kramer BK, Kurtz A (2001). Functional role of sodium–calcium exchange in the regulation of renal vascular resistance. *Am J Physiol Renal Physiol* **280**: F155–F161.

Sedova M, Blatter LA (1999). Dynamic regulation of $[Ca^{2+}]_i$ by plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange during capacitative Ca^{2+} entry in bovine vascular endothelial cells. *Cell Calcium* **25**: 333–343.

Shelkovnikov S, Merlic CA, Gonick HC (2004). Influence of nitric oxide donors and peroxynitrite on the contractile effect and concentration of norepinephrine. *Life Sci* **74**: 2919–2928.

Sofola O, Yakubu M, Igbo I, Newaz M, Oyekan A (2003). High salt diet modulates cAMP- and nitric oxide-mediated relaxation responses to isoproterenol in the rat aorta. *Eur J Pharmacol* **474**: 241–247.

Streefkerk JO, de Groot AA, Pfaffendorf M, van Zwieten PA (2002). Influence of the nature of pre-contraction on the responses to commonly employed vasodilator agents in rat-isolated aortic rings. *Fundam Clin Pharmacol* **16**: 485–494.

Takai N, Yamada A, Muraki K, Watanabe M, Imaizumi Y (2004). KB-R7943 reveals possible involvement of Na^+/Ca^{2+} exchanger in elevation of intracellular Ca^{2+} in rat carotid arterial myocytes. *J Smooth Muscle Res* **40**: 35–42.

Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, Shigenobu K (2002). Effect of SEA0400, a novel inhibitor of sodium–calcium exchanger, on myocardial ionic currents. *Br J Pharmacol* **135**: 1096–1100.

Teubl M, Groschner K, Kohlwein SD, Mayer B, Schmidt K (1999). Na(+)/Ca(2+) exchange facilitates Ca(2+)-dependent activation of endothelial nitric-oxide synthase. *J Biol Chem* **274**: 29529–29535.

Toma, Serban DN, Costache V, Branisteanu DD (1995). Characteristics and possible mechanisms of low-Na^+ induced contractions in rat aorta. *Naunyn Schmiedebergs Arch Pharmacol* **352**: 88–93.

van der Zypp A, Kang KB, Majewski H (2000). Age-related involvement of the endothelium in beta-adrenoceptor-mediated relaxation of rat aorta. *Eur J Pharmacol* **397**: 129–138.

Vane J, Botting R (1987). Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB J* **1**: 89–96.

Vargas F, Osuna A (1996). Modulatory role of endothelium-derived relaxing factors on the response to vasoconstrictors and flow-pressure curve in the isolated perfused rat kidney. *J Vasc Res* **33**: 119–123.

Wang X, Reznick S, Li P, Liang W, van Breemen C (2002). Ca(2+) removal mechanisms in freshly isolated rabbit aortic endothelial cells. *Cell Calcium* **31**: 265–277.

Watano T, Kimura J, Morita T, Nakanishi H (1996). A novel antagonist, No. 7943, of the Na^+/Ca^{2+} exchange current in guinea-pig cardiac ventricular cells. *Br J Pharmacol* **119**: 555–563.

Wedel BJ, Garbers DL (1997). New insights on the functions of the guanylyl cyclase receptors. *FEBS Lett* **410**: 29–33.

Wei GZ, Zhou JJ, Wang B, Wu F, Bi H, Wang YM *et al*. (2007). Diastolic Ca^{2+} overload caused by Na^+/Ca^{2+} exchanger during the first minutes of reperfusion results in continued myocardial stunning. *Eur J Pharmacol* **572**: 1–11.

Wu BN, Chen IC, Lin RJ, Chiu CC, An LM, Chen IJ (2005). Aortic smooth muscle relaxants KMUP-3 and KMUP-4, two nitrophenylpiperazine derivatives of xanthine, display cGMP-enhancing activity: roles of endothelium, phosphodiesterase, and K^+ channel. *J Cardiovasc Pharmacol* **46**: 600–608.

Yamanaka J, Nishimura J, Hirano K, Kanaide H (2003). An important role for the Na^+–Ca^{2+} exchanger in the decrease in cytosolic Ca^{2+} concentration induced by isoprenaline in the porcine coronary artery. *J Physiol* **549**: 553–562.

Yang Z, Zhang A, Altura BT, Altura BM (1999). Hydrogen peroxide-induced endothelium-dependent relaxation of rat aorta involvement of Ca^{2+} and other cellular metabolites. *Gen Pharmacol* **33**: 325–336.

Zhao J, Murphy TV, Hill MA, Majewski H (2004). The Na^+/Ca^{2+} exchanger contributes to the regulation of myogenic tone in rat arterioles. *Clin Exp Physiol Pharmacol* **31** (Suppl 1): A192.