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Evidence that different mechanisms underlie smooth muscle relaxation to nitric oxide and nitric oxide donors in the rabbit isolated carotid artery

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1 The endothelium-dependent relaxants acetylcholine (ACh; 0.03–10 μM) and A23187 (0.03–10 μM), and nitric oxide (NO), applied either as authentic NO (0.01–10 μM) or as the NO donors 3-morpholino-sydnonimine (SIN-1; 0.1–10 μM) and S-nitroso-N-acetylpenicillamine (SNAP; 0.1–10 μM), each evoked concentration-dependent relaxation in phenylephrine stimulated (1–3 μM; mean contraction and depolarization, 45.8±5.3 mV and 31.5±3.3 mN; $n=10$) segments of rabbit isolated carotid artery. In each case, relaxation closely correlated with repolarization of the smooth muscle membrane potential and stimulated a maximal reversal of around 95% and 98% of the phenylephrine-induced depolarization and contraction, respectively.

2 In tissues stimulated with 30 mM KCl rather than phenylephrine, smooth muscle hyperpolarization and relaxation to ACh, A23187, authentic NO and the NO donors were dissociated. Whereas the hyperpolarization was reduced by 75–80% to around a total of 10 mV, relaxation was only inhibited by 35% ($n=4–7$ in each case; $P<0.01$). The responses which persisted to ACh and A23187 in the presence of 30 mM KCl were abolished by either the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME; 100 μM) or the inhibitor of soluble guanylyl cyclase 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM; 10 min; $n=4$ in each case; $P<0.01$).

3 Exposure to ODQ significantly attenuated both repolarization and relaxation to ACh, A23187 and authentic NO, reducing the maximum changes in both membrane potential and tension to each relaxant to around 60% of control values ($n=4$ in each case; $P<0.01$). In contrast, ODQ almost completely inhibited repolarization and relaxation to SIN-1 and SNAP, reducing the maximum responses to around 8% in each case ($n=3–5$; $P<0.01$).

4 The potassium channel blockers glibenclamide (10 μM), iberiotoxin (100 nM) and apamin (50 nM), alone or in combination, had no significant effect on relaxation to ACh, A23187, authentic NO, or the NO donors SIN-1 and SNAP ($n=4$ in each case; $P>0.05$). Charybdotoxin (ChTX; 50 nM) almost abolished repolarization to ACh ($n=4$; $P<0.01$) and inhibited the maximum relaxation to ACh, A23187 and authentic NO each by 30% ($n=4–8$; $P<0.01$). Application of ODQ (10 μM; 10 min) abolished the ChTX-insensitive responses to ACh, A23187 and authentic NO ($n=4$ in each case; $P<0.01$

5 When the concentration of phenylephrine was reduced (to 0.3–0.5 μM) to ensure the level of smooth muscle contraction was the same as in the absence of potassium channel blocker, ChTX had no effect on the subsequent relaxation to SIN-1 ($n=4$; $P>0.05$). However, in the presence of tone induced by 1–3 μM phenylephrine (51.2±3.3 mN; $n=4$), ChTX significantly reduced relaxation to SIN-1 by nearly 50% (maximum relaxation 53.2±6.3%, $n=4$; $P<0.01$).

6 These data indicate that NO-evoked relaxation of the rabbit isolated carotid artery can be mediated by three distinct mechanisms: (a) a cyclic GMP-dependent, voltage-independent pathway, (b) cyclic GMP-mediated smooth muscle repolarization and (c) cyclic GMP-independent, ChTX-sensitive smooth muscle repolarization. Relaxation and repolarization to both authentic and endothelium-derived NO in this large conduit artery appear to be mediated by parallel cyclic GMP-dependent and -independent pathways. In contrast, relaxation to the NO-donors SIN-1 and SNAP appears to be mediated entirely via cyclic GMP-dependent mechanisms.

Keywords: Nitric oxide; smooth muscle relaxation; potassium channels

Introduction

Endothelium-dependent relaxation of isolated resistance arteries is mediated by the release of both nitric oxide (NO) and a factor distinct from NO termed endothelium-derived hyperpolarizing factor (EDHF; for review see Garland *et al*., 1995). We have recently shown that, in contrast to small resistance vessels, endothelium-dependent relaxation and repolarization of the rabbit isolated carotid artery can be fully accounted for by the release of endothelium-derived NO, with little or no contribution from EDHF (Cohen *et al*., 1997). However, although there is a good correlation between NO release, smooth muscle repolarization and relaxation to both ACh and the NO donor 3-morpholino-sydnonimine (SIN-1) in this artery, the mechanisms underlying the changes in smooth muscle membrane potential and tone to both endothelium-derived and exogenous NO have not been determined.

NO can stimulate soluble guanylyl cyclase in smooth muscle cells to increase the second messenger guanosine 3′:5′-cyclic monophosphate (cyclic GMP), which is thought to lead to relaxation largely via voltage-independent mechanisms. Recently, NO has been shown to cause hyperpolarization of the smooth muscle cell resting membrane potential and repolarization of the depolarized membrane potential, in a number of

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isolated arteries (Tare *et al*., 1990; Garland & McPherson, 1992; Plane *et al*., 1995; Cohen *et al*., 1997). In addition, cyclic GMP and NO (either directly or via cyclic GMP) can activate calcium-dependent, charybdotoxin (ChTX)-sensitive potassium channels in freshly isolated smooth muscle cells (Robertson *et al*., 1993; Bolotina *et al*., 1994; George & Shibata, 1995). Furthermore, a recent study has shown that, in rat endothelium-intact isolated mesenteric resistance arteries, full relaxation to the NO donor SIN-1 can be accounted for by a ChTX-sensitive, cyclic GMP-independent mechanism (Plane *et al*., 1996a).

In the present study, the mechanisms underlying smooth muscle repolarization and relaxation to both endothelium-derived and exogenous NO were examined in the rabbit isolated carotid artery. Preliminary accounts of this study have been presented to the British Pharmacological Society (Plane *et al*., 1996b; 1997).

Methods

Female New Zealand White rabbits (2–3 kg) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.v.). The carotid arteries were carefully removed, cleaned of adhering fat and connective tissue and cut into cylindrical segments. Arterial segments were mounted in a Mulvany-Halpern myograph under normalized tension as previously described (Cohen *et al*., 1997). Briefly, the segments of artery were mounted on two tungsten wires (40 μm in diameter) and the segments attached to the feet of the myograph. Arterial segments were maintained in a static bath, at 37°C, in oxygenated Krebs buffer containing indomethacin (1 μM).

Cumulative concentration-response curves to the relaxants were constructed in arterial segments depolarized and preconstricted with phenylephrine (phenylephrine; 1–3 μM). In the presence of inhibitors, the concentration of phenylephrine used to induce arterial constriction was adjusted to give a similar level of tone to control experiments.

Electrophysiology

Measurement of smooth muscle membrane potential was made with a glass microelectrode advanced through the adventitial surface of the arterial segment. The electrodes were filled with 2 M KCl and had resistances of 60–120 MΩ. Membrane electrical events were recorded through a high impedance d.c. pre-amplifier (Neurolog 102G) and together with data from the isometric force transducer, stored on disc (MacLab, AD Instruments).

Measurement of cyclic GMP levels

In a parallel study, increases in tissue cyclic GMP levels in 2 mm long segments of carotid artery were measured by radioimmunoassay (Amersham International) in the presence of isobutylmethylxanthine as previously described (Miller *et al*., 1994). Tissues were incubated with phenylephrine (3 μM) and either SIN-1 (10 μM) or ACh (10 μM), for 15 min.

Solutions and drugs

Tissues were maintained in Krebs buffer of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.18, glucose 11, disodium EDTA 0.027 and $CaCl_2$ 2.5. Buffer containing raised concentrations of KCl was prepared by equimolar substitution of NaCl. Drugs used were from Sigma except for SIN-1, S-nitroso-N-acetylpenicillamine (SNAP) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris) and ChTX (CalBiochem). All drugs were dissolved in Krebs buffer except for A23187, ODQ and SNAP, which were dissolved in DMSO.

Preparation of NO solutions

NO gas (research grade BDH) was injected into sealed vials containing Krebs solution which had been bubbled with helium or argon for 45–60 min at 4°C. NO solutions were injected from a gas-tight syringe close to the artery segments, in volumes of less than 250 μl. Control injections of Krebs solution were made to assess the extent of any injection artefacts.

Analysis of data

Arterial relaxations are expressed as the percentage decrease in the induced level of tone and smooth muscle repolarization is expressed either in mV or as the percentage reversal of depolarization. EC_{50} values are expressed as pD_2 values ($-\log EC_{50}$). All data are expressed as mean ± s.e.mean and the significance of differences between mean values was calculated by Student's *t* test or by ANOVA.

Results

Membrane potential and tension responses to ACh and A23187

Smooth muscle cells in isolated segments of the carotid artery had a mean resting membrane potential of -56.7 ± 4.6 mV ($n = 70$ cells from 30 preparations). Phenylephrine (1–3 μM) stimulated depolarization and contraction of the arterial segments (mean depolarization and contraction 45.8 ± 5.3 mV and 31.5 ± 3.3 mN; $n = 10$) which were concentration-dependently reversed by ACh (0.03–10 μM) or A23187 (0.03–10 μM). A representative trace showing simultaneous measurements of changes in tension and membrane potential to ACh is shown in Figure 1a. The threshold concentrations and pD_2 values for the repolarization and relaxation evoked by each relaxant were not significantly different (0.01 μM, 6.90 ± 0.12 and 7.12 ± 0.09, and 0.05 μM, 6.90 ± 0.15 and 6.90 ± 0.10, respectively; $n = 24$ and 5; $P > 0.05$). The maximum reversal of depolarization and contraction to each relaxant was $97.6 \pm 4.1\%$ and $98.0 \pm 5.3\%$ ($n = 24$), and $93.9 \pm 4.2\%$ and $97.8 \pm 4.4\%$ ($n = 5$), respectively.

Effect of KCl on membrane and tension responses to ACh and A23187

Prestimulation of arterial segments with 30 mM KCl induced similar smooth muscle depolarization and contraction as 1–3 μM phenylephrine (48.2 ± 5.0 mV and 35.8 ± 4.4 mN; $n = 10$; $P > 0.05$). As shown in Figure 1b and c, in the presence of 30 mM KCl, the reversal of depolarization by both ACh and A23187 was reduced by around 80%, to only 5.6 ± 4.1 mV and 7.5 ± 4.5 mV, respectively ($n = 4$ in each case; $P < 0.01$). In contrast, the relaxation with each of these agents was only reduced by about 35% to $63.6 \pm 5.5\%$ and $63.1 \pm 6.4\%$, respectively ($n = 5$ in each case; $P < 0.01$). Increasing the concentration of KCl to 40 mM abolished the remaining repolarization and relaxation to ACh and A23187 ($n = 4$ in each case; $P < 0.01$).

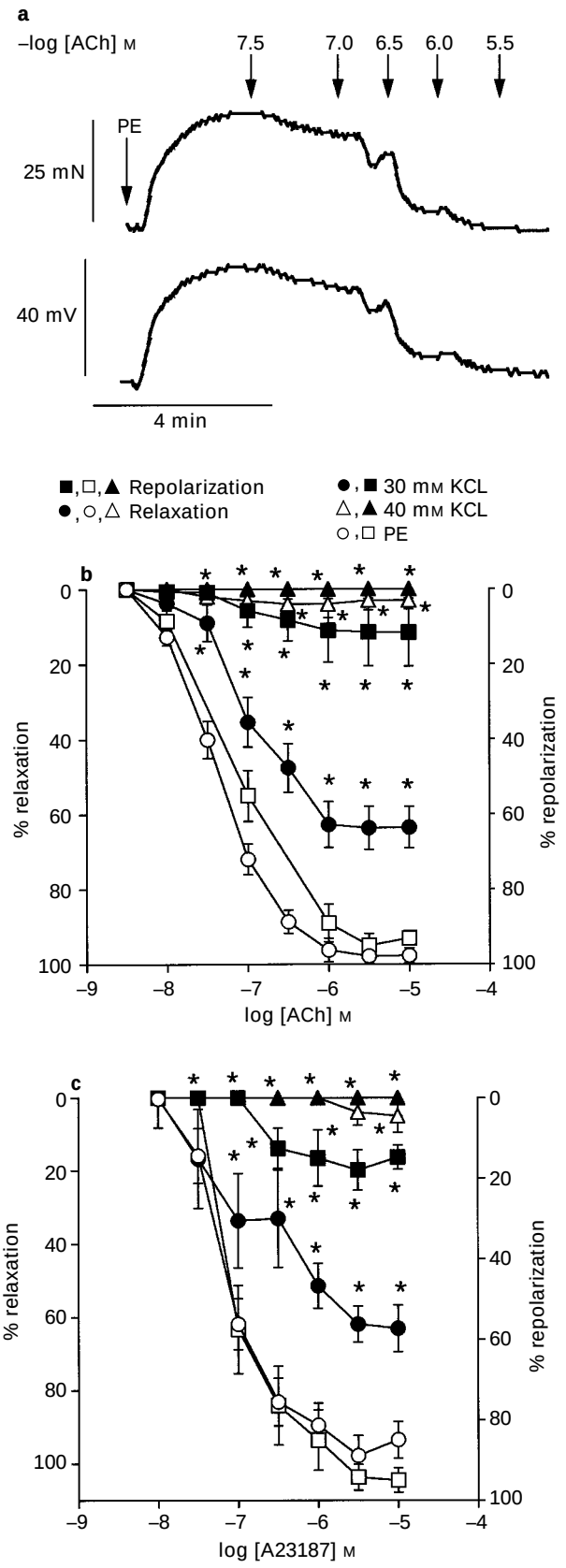

Figure 1 (a) Representative traces showing simultaneous recording of ACh (0.03–10 μM)-evoked changes in smooth muscle membrane potential and tension in arterial segments stimulated with phenylephrine (3 μM). (b and c) Mean concentration-response curves for (b) ACh- and (c) A23187-evoked repolarization and relaxation in tissues stimulated with 30 mM KCl, 40 mM KCl or phenylephrine (PE; 1–3 μM). All points are means of 4 to 5 experiments with s.e.means shown by vertical lines. *$P<0.01$ compared to control values.

Role of cyclic GMP in repolarization and relaxation to ACh and A23187

Concentration-response curves for repolarization and relaxation to ACh in the presence and absence of ODQ (10 μM; 10 min), an inhibitor of soluble guanylyl cyclase, are shown in Figure 2a. Pre-incubation with ODQ significantly and similarly attenuated the repolarization and relaxation to both ACh and A23187, reducing the maximum responses by close to 40% (28.5 ± 2.3 mV and 62.1 ± 7.1%, and 29.8 ± 3.0 mV and 63.4 ± 11.6%, respectively: $n=4$ in each case; $P<0.01$). The relaxation to both ACh and A23187 which remained in the presence of ODQ was abolished by either the NO synthase inhibitor L-NAME (30 μM) or 30 mM KCl ($n=4$ in each case; $P<0.05$).

In parallel experiments, this concentration of ODQ was found to abolish the increases in cyclic GMP caused by ACh ($n=6$; $P<0.01$; Figure 2b) or A23187 (data not shown).

The effect of potassium channel blockers on membrane potential and tension changes to ACh and A23187

The potassium channel inhibitors glibenclamide (10 μM), iberiotoxin (IbTX; 100 nM) and apamin (50 nM), alone or in combination, had no effect on smooth muscle relaxation to either ACh (Figure 2c) or A23187 ($n=4$ in each case; $P>0.05$). ChTX (50 nM) abolished the repolarization to ACh but only reduced the relaxation to ACh and A23187 by around 30% (maximal relaxations 75.0 ± 10.7% and 68.0 ± 7.5%; $n=4$ in each case; $P<0.01$). However, in the presence of ODQ (10 μM; 10 min), a concentration which blocked increases in cyclic GMP, repolarization and relaxation to ACh and A23187 were abolished by ChTX ($n=4$ in each case; $P<0.01$; Figure 2d). Exposure to apamin or iberiotoxin together with ODQ did not cause any further reduction in the relaxation to ACh compared with ODQ alone ($n=4$; $P>0.05$; Figure 2d).

Membrane and tension responses to authentic NO and the NO donors SIN-1 and SNAP

Authentic NO (0.01–10 μM) and the NO donors, SIN-1 (0.1–10 μM) and SNAP (0.1–10 μM), each caused concentration-dependent relaxation of arterial segments prestimulated with phenylephrine (1–3 μM). The relaxation was closely correlated with smooth muscle cell repolarization and a representative trace showing simultaneous measurements of changes in membrane potential and tension with SIN-1 is shown in Figure 3a. Whereas the responses to the NO donors were sustained, changes in membrane potential and tension to authentic NO were transient. As shown in Table 1, the threshold and pD_2 values for the repolarization and relaxation evoked by authentic NO and SIN-1 were not significantly different and neither the repolarization nor relaxation to either NO or SIN-1 was altered by the removal of the endothelium, or by incubating endothelium-intact arterial segments with L-NAME (100 μM; $n=4$ in each case; $P>0.05$). However, preincubation with superoxide dismutase (SOD; 30 u ml^{-1}) significantly potentiated relaxation to both SIN-1 and authentic NO ($n=5$; $P<0.01$; Table 1).

Effect of 30 mM KCl on membrane and tension responses to NO and NO donors

As shown in Figure 3b, in tissues prestimulated with 30 mM KCl, hyperpolarization to NO was inhibited by around 80%, whereas the NO-evoked relaxation was only reduced by 50%

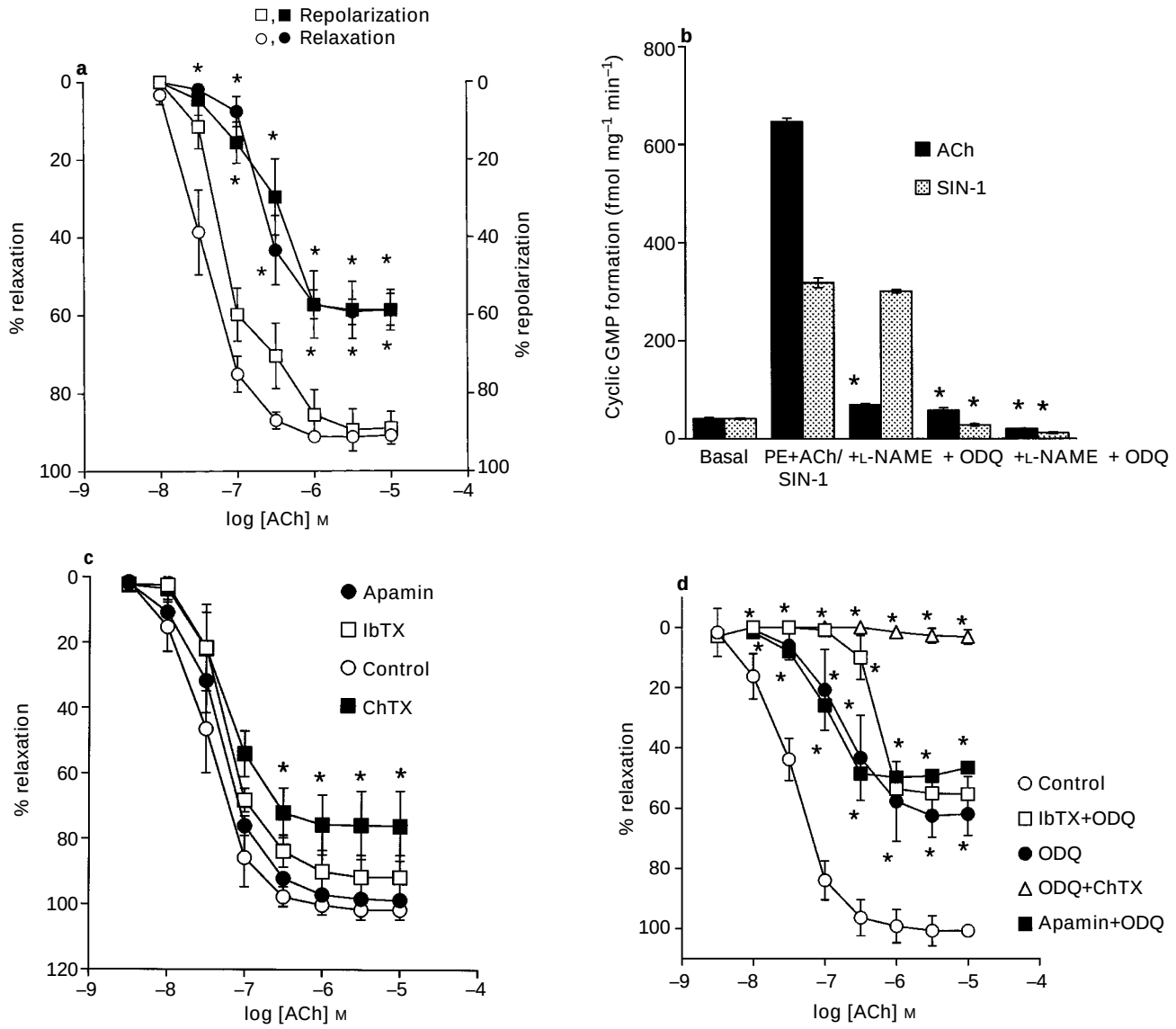

Figure 2 (a) Mean concentration-response curves for ACh-evoked repolarization and relaxation in the presence (solid symbols) and absence (open symbols) of ODQ (10 μM). (b) Effect of ODQ (10 μM) and L-NAME (100 μM) on mean increases in cyclic GMP stimulated by ACh (10 μM; $n=6$) and SIN-1 (10 μM; $n=6$). (c) Mean concentration-response curves for ACh-evoked relaxation in the presence and absence of apamin (500 nM), IbTX (100 nM) and ChTX (50 nM). (d) Mean concentration-response curves for ACh-evoked relaxation in the presence and absence of ODQ (10 μM), IbTX and ODQ, apamin and ODQ, ChTX and ODQ. All points are means of 4–6 experiments with s.e.means shown by vertical lines. *$P<0.01$ compared to control values.

(maximum hyperpolarization and relaxation 9.5 ± 2.5 mV and $51.0\pm2.3\%$, respectively; $n=3$ and 5; $P<0.01$). Similarly, both hyperpolarization and relaxation to SIN-1 and SNAP were attenuated in arterial segments precontracted with 30 mM KCl (Figure 3c). The maximal hyperpolarization to each agent was reduced by close to 75% (maximal hyperpolarization 9.8 ± 2.8 mV and 10.9 ± 5.8 mV, respectively; $n=4$ and 3; $P<0.01$), whereas the maximal relaxation was only reduced by around 35% (maximal relaxation $60.3\pm8.7\%$ and $68.8\pm5.1\%$, respectively; $n=7$ and 4; $P<0.01$).

Role of cyclic GMP in repolarization and relaxation of NO and NO donors

Preincubation with ODQ (10 μM; 10 min) partially blocked both the repolarization and relaxation to authentic NO, reducing the maximum responses to 25.2 ± 4.0 mV and $64.9\pm5.2\%$ ($n=3$ and 6; $P<0.01$; Figure 4a), respectively. In contrast, ODQ almost completely inhibited both the repolarization and relaxation to both SIN-1 and SNAP, in these cases reducing the maximum change in membrane potential and tension to each agent to 3.0 ± 2.5 mV and $6.0\pm1.5\%$, 4.2 ± 4.0 mV and $9.9\pm6.8\%$, respectively ($n=4$ in each case; $P<0.01$; Figure 4b). The inhibitory effect of ODQ on relaxation to both authentic NO and SIN-1 was not altered by the presence of SOD (30 u ml^{-1}; $n=3$ in each case; $P>0.05$). In parallel experiments, ODQ (10 μM) abolished SIN-1-evoked increases in cyclic GMP accumulation (Figure 2b) ($n=6$; $P<0.01$).

Effect of potassium channel blockers on repolarization and relaxation to NO and NO donors

The potassium channel blockers glibenclamide (10 μM), apamin (50 nM) and IbTX (100 nM), alone or in combination, did not significantly alter the smooth muscle relaxation to either NO, SIN-1 or SNAP ($n=4$ in each case; $P>0.05$; Figure 5a and b). ChTX (50 nM) had no effect on relaxation to the NO donor SIN-1 ($n=4$; $P>0.05$; Figure 5b), but did

attenuate the relaxation to authentic NO, reducing the maximum relaxation by around 30% to 69.4±5.8% ($n=6$; $P<0.01$; Figure 5a). The relaxation to NO which remained in the presence of ChTX was abolished by ODQ (10 μM; $n=4$; $P<0.01$; Figure 5a).

Depolarization and contraction to phenylephrine were significantly potentiated in the presence of ChTX. Thus, in the experiments described above, the concentration of phenylephrine used to prestimulate the arterial segments was reduced to between 0.3–0.5 μM. However, if the concentration of phenylephrine used in control experiments, 1–3 μM, was applied in the presence of ChTX (maximum contraction 51.2±3.3 mN; $n=4$), a significant inhibition of the SIN-1-evoked relaxation was obtained. The maximum response was reduced to 53.2±6.3% ($n=4$; $P<0.01$). These inhibitory actions of ChTX were not mimicked by the other potassium channel inhibitors ($n=4$ in each case; $P>0.05$), and increasing the level of tone with phenylephrine in the absence of ChTX did not significantly alter SIN-1-evoked responses ($n=4$; $P>0.05$).

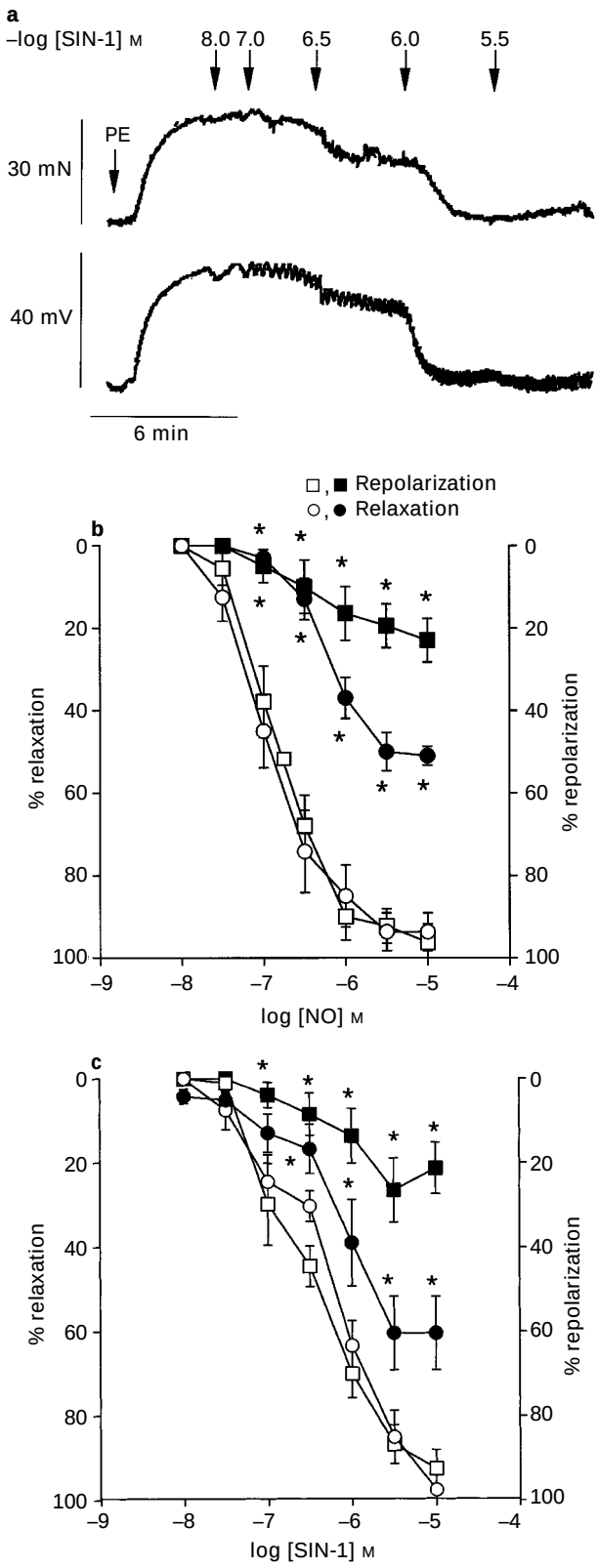

Figure 3 (a) Representative traces showing simultaneous recording of SIN-1 (0.1–3 μM)-evoked changes in smooth muscle membrane potential and tension in arterial segments stimulated with phenylephrine (3 μM). (b and c) Mean concentration-response curves for (b) NO- and (c) SIN-1-evoked repolarization and relaxation in the presence (solid symbols) and absence (open symbols) of 30 mM KCl. All points are means of 4 to 5 experiments with s.e.means shown by vertical lines. *$P<0.01$ compared to control values.

Discussion

In the present study, the endothelium-dependent relaxants ACh and the calcium ionophore A23187, each evoked concentration-dependent repolarization and relaxation of isolated arterial segments depolarized and constricted with phenylephrine. The pattern of inhibition of these responses by NO synthase inhibitors supports previous observations with this artery (Cohen *et al.*, 1997), and is consistent with our proposal that NO mediates both the endothelium-dependent repolarization and relaxation to ACh and A23187 in this vessel. Solutions of authentic NO and the NO donors SIN-1 and SNAP also each evoked concentration-dependent relaxation of phenylephrine-stimulated tissues, which in each case was closely accompanied by repolarization of the smooth muscle cell membrane potential. However, when smooth muscle repolarization was inhibited in the presence of 30 mM KCl, smooth muscle relaxation to both endothelium-derived and authentic NO, and to the NO donors, was only reduced by around 35%. Thus, although relaxation of the rabbit isolated carotid artery to both endothelium-derived and exogenous NO is closely mirrored by smooth muscle repolarization, other mechanisms can account for most of the relaxation in cells contracted and depolarized with phenylephrine.

Although the experiments carried out with 30 mM KCl indicated that similar pathways may underlie relaxation to both endothelium-derived and exogenous NO in the carotid artery, studies carried out with the inhibitor of soluble guanylyl cyclase, ODQ (Garthwaite *et al.*, 1995) revealed marked differences in the contribution of cyclic GMP to the repolarization and relaxation. When increases in cyclic GMP were blocked, as confirmed by radioimmunoassay, both the changes in tension and membrane potential induced by the NO donors were almost abolished. This indicates that both components of the response to these agents can be fully accounted for by cyclic GMP-dependent pathways.

In contrast, around 60% of the repolarization and relaxation to both authentic and endothelium-derived NO persisted in the presence of ODQ. These cyclic GMP-independent responses were abolished in the presence of 30 mM KCl. Thus, in contrast to the NO donors, a large component of relaxation to both endothelium-derived and authentic NO is mediated by cyclic GMP-independent smooth muscle repolarization. This cyclic GMP-independent repolarization may reflect a direct action of NO on potassium channels, as has been demonstrated in isolated smooth muscle cells (Bolotina *et al.*, 1994).

Table 1 Effect of endothelial removal, L-NAME and SOD on relaxation and repolarization to SIN-1 and authentic NO

	SIN-1		*NO*	
	Relaxation	*Repolarization*	*Relaxation*	*Repolarization*
Control				
Threshold concentration	0.05 μM	0.05 μM	0.05 μM	0.05 μM
R_{max}	95.3±4.5%	92.4±3.2%	93.7±4.6%	96.3±4.5%
pD_2	6.3±0.10 μM	6.4±0.14 μM	6.7±0.18 μM	6.7±0.15 μM
Endothelium-denuded				
Threshold concentration	0.05 μM	0.05 μM	0.05 μM	0.05 μM
R_{max}	96.0±5.2%	94.6±3.1%	95.4±4.0%	93.2±4.1%
pD_2	6.2±0.20 μM	6.3±0.20 μM	6.8±0.24 μM	6.8±0.10 μM
+L-NAME (100 μM)				
Threshold concentration	0.05 μM	0.05 μM	0.05 μM	0.05 μM
R_{max}	94.8±3.1%	92.1±6.2%	95.8±1.5%	95.0±4.8%
pD_2	6.2±0.25 μM	6.2±0.16 μM	6.7±0.3 μM	6.8±0.20 μM
+SOD (30 u ml^{-1})				
Threshold concentration	0.01 μM*	ND	0.01 μM*	ND
R_{max}	98.4±4.3%	ND	99.0±1.7%	ND
pD_2	7.3±0.10 μM*	ND	7.6±0.18 μM*	ND

ND = not determined, R_{max} = maximum response, *denotes $P < 0.01$ compared to control.

To date there are no other studies indicating that direct activation of potassium channels can underlie arterial vasodilatation to NO. However, in the rabbit isolated aorta relaxation to endothelium-derived NO, in the presence of methylene blue, has been shown to be inhibited by ChTX, indicating that this mechanism may contribute to NO-induced smooth relaxation in other vessels (Bolotina *et al*., 1994). In small resistance arteries, the role of potassium channels in relaxation to endothelium-derived NO has been difficult to elucidate, as endothelium-dependent hyperpolarization is largely mediated by EDHF in these vessels (Garland *et al*., 1995). However, dilatation of the rat isolated perfused mesenteric bed to the nitrovasodilator glyceryl trinitrate was not abolished by methylene blue (Khan *et al*., 1993a) and a recent study has demonstrated that in rat isolated mesenteric arteries relaxation to SIN-1 can be fully accounted for by an ODQ-insensitive, ChTX-sensitive pathway (Plane *et al*., 1996a). Thus, the role of cyclic GMP-independent activation of potassium channels in relaxation to both endothelium-derived and exogenous NO may vary between vessels and requires further investigation.

The importance of these data is two fold. Firstly, they demonstrate that three mechanisms can contribute to NO-evoked relaxation in the rabbit carotid artery; a cyclic GMP-mediated, voltage-independent mechanism, cyclic-GMP-dependent smooth muscle repolarization and cyclic GMP-independent repolarization. Secondly, the contribution the individual pathways make to smooth muscle relaxation in response to NO is different, depending on the source of the NO, i.e. whether it is released by the endothelium, applied as a gas in solution or released from NO-donors.

The reason for the differences in the mechanisms which underlie relaxation to NO from different sources is unclear. However, the relaxation to SIN-1 and SNAP was much slower than the changes in tension to either endothelium-derived or authentic NO. Indeed, the difference in the time course of the response reflects a much slower release of NO from SIN-1, which requires 4 min to stabilize after addition to Krebs solution compared with the peak concentration of NO released by ACh which peaks within 2 s (Cohen *et al*., 1997). Thus, differences in the mechanism of action may reflect a variation in the rate at which NO is released or gains access and accumulates in the cytoplasm of the smooth muscle cells.

In addition to releasing NO, SIN-1 also generates superoxide which may interact with NO to form peroxynitrite and its degradation products, such as hydroxyl radical and nitrogen dioxide, all of which can relax arterial smooth muscle (Feelisch, 1991; Hogg *et al*., 1992). However, in the present study, addition of SOD significantly potentiated relaxation to both the NO donors and authentic NO, but did not alter the mechanisms mediating relaxation to these agents. This indicates that the interaction of NO with superoxide does not contribute significantly to the differences between the modes of action of these agents in the carotid artery.

Which specific type of potassium channel is activated by NO is unclear, and indeed appears to vary between different blood vessels and species. Hyperpolarization of the resting membrane potential of rat and rabbit mesenteric arteries to NO (Garland & McPherson, 1992; Murphy & Brayden, 1995) and NO-evoked repolarization of noradrenaline-stimulated segments of rabbit isolated femoral artery (Plane *et al*., 1995) was abolished by glibenclamide, indicating a role for ATP-sensitive potassium channels in the NO-evoked response. Activation of glibenclamide-sensitive potassium channels may also contribute to NO-induced dilatation of cerebral arteries (Armstead, 1996). However, in the present study and a number of other studies (Khan *et al*., 1993b; Plane *et al*., 1996a,b; Zhao *et al*., 1997), relaxation to NO from a number of sources was not modified by glibenclamide. Similarly, although activation of apamin-sensitive, small conductance calcium-activated potassium channels has been associated with NO-evoked relaxation and hyperpolarization in a number of smooth muscle preparations (Ward *et al*., 1992; Watson *et al*., 1996), in the present study, as in rat and rabbit mesenteric arteries (Murphy & Brayden, 1995; Plane *et al*., 1996a) apamin did not affect relaxation to NO from any of the different sources.

Recently, both NO and cyclic GMP have been shown to activate ChTX-sensitive potassium channels in isolated smooth muscle cells (Robertson *et al*., 1993; Bolotina *et al*., 1994) and ChTX has been shown to inhibit NO and cyclic GMP-induced relaxation (Khan *et al*., 1993b; Bialecki & Stinson-Fisher, 1995; Archer *et al*., 1995; Plane *et al*., 1996a,b). We found that ChTX has very similar effects on NO-evoked responses as raising the extracellular concentration of KCl. That is, repolarization was abolished and relaxation to both endothelium-derived and authentic NO was attenuated by around 30%. However, in the presence of ODQ, which we

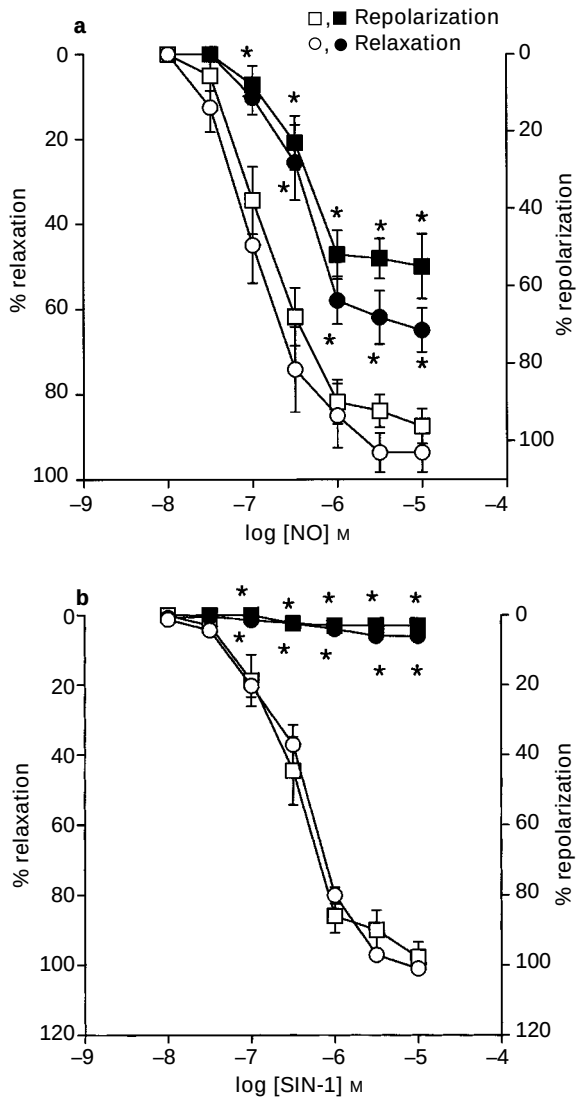

Figure 4 Mean concentration-response curves for (a) NO- and (b) SIN-1-evoked repolarization and relaxation in the presence (solid symbols) and absence (open symbols) of ODQ (10 μM). All points are means of 4 experiments with s.e.means shown by vertical lines. $^*P<0.01$ compared to control values.

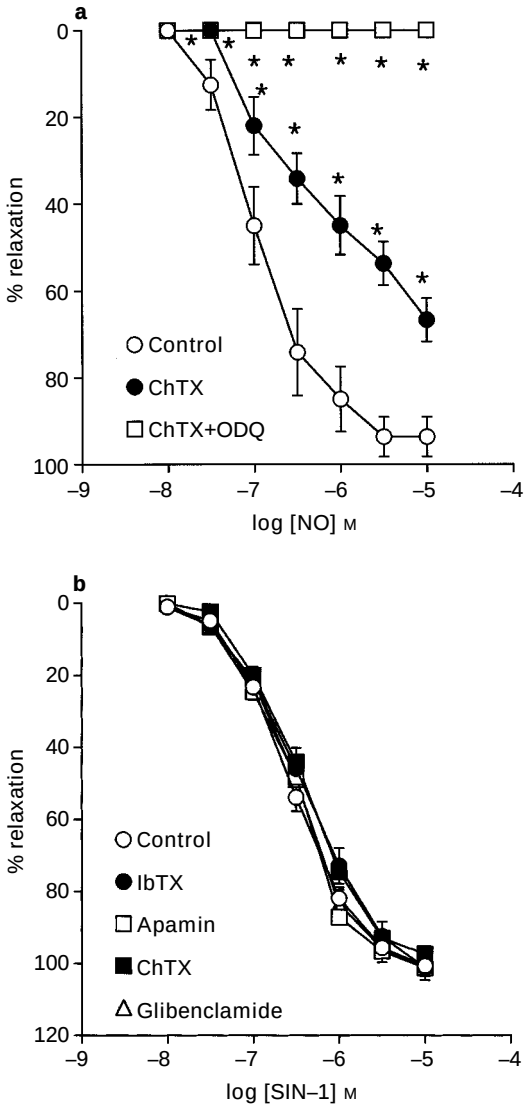

Figure 5 Mean concentration-relaxation curves for (a) NO in the absence and presence of ChTX (50 nM) and ChTX and ODQ. (b) SIN-1 in the absence and presence of IbTX (100 nM), apamin (50 nM), ChTX (50 nM) and glibenclamide (5 μM). All points are means of 4 experiments with s.e.means shown by vertical lines. $^*P<0.01$ compared to control values.

know blocked the synthesis of cyclic GMP, both the ODQ-insensitive repolarization and relaxation to endothelium-derived and authentic NO was abolished by ChTX. This indicates that cyclic GMP-independent membrane repolarization to NO is most likely mediated by the activation of ChTX-sensitive potassium channels in the carotid artery, supporting the observations of Bolotina *et al.* (1994). A recent study has suggested that in hypercholesterolaemic rabbits, cyclic GMP-mediated relaxation of the carotid artery is impaired, but that normal smooth muscle relaxation to both endothelium-derived and authentic NO is maintained via activation of ChTX-sensitive potassium channels (Najibi *et al.*, 1994; Najibi & Cohen, 1995). Therefore, under normal conditions around 70% of the ability of NO to induce relaxation persists in the absence of a change in smooth muscle membrane potential, but under pathophysiological conditions cyclic GMP-independent repolarization may provide the major route for arterial dilatation.

In contrast to endothelium-derived and authentic NO, none of the potassium channel blockers used in this study, either alone or in combination, inhibited relaxation to the NO donors. The experiments carried out with raised KCl suggest that, as with endothelium-derived and authentic NO, around 30% of the relaxation to these agents is mediated by membrane repolarization. However, the lack of effect of the potassium channel blockers indicates that another type of channel, possibly a voltage-sensitive potassium channel, might underlie this component of the relaxation. Both the depolarization and contraction to phenylephrine were significantly potentiated in the presence of the potassium channel blockers. Therefore, in these experiments the concentration of phenylephrine used to prestimulate the arterial segments was reduced. However, when the concentration range employed in the absence of the blockers (1–3 μM) was applied in the presence of ChTX, a significant inhibition of SIN-1-evoked relaxation was obtained. Under these conditions, the maximal response was reduced by about 50%.

The potentiation of phenylephrine-induced contraction observed in the presence of ChTX may be due to potassium channel blockade, causing smooth muscle depolarization and

increased calcium influx through voltage-sensitive calcium channels (Brayden & Nelson, 1992). Increasing the level of depolarization and tone with phenylephrine, in the absence of ChTX, did not significantly alter SIN-1-evoked responses. Thus the inhibitory effect of ChTX may be due to a non-specific effect of ChTX on the contractile mechanisms which are activated by phenylephrine. In addition to inhibiting large conductance calcium-activated potassium channels, ChTX also inhibits delayed rectifier channels (Kaczorowski *et al*., 1996). Therefore, the lack of effect of iberiotoxin, a more selective inhibitor of large conductance potassium channels, may indicate that it is in fact these channels which are involved in NO-induced changes in tone rather than the large conductance potassium channels (Zhao *et al*., 1997).

In conclusion, these data indicate that NO-evoked relaxation of the rabbit isolated carotid artery can be mediated by three distinct mechanisms: (a) a cyclic GMP-dependent, voltage-independent pathway, (b) cyclic GMP-mediated smooth muscle repolarization and (c) cyclic GMP-independent, ChTX-sensitive smooth muscle repolarization. However, the relative contribution of these pathways to changes in tone is different depending on the source of NO. Parallel cyclic GMP-dependent and -independent pathways appear to mediate both relaxation and repolarization to authentic and endothelium-derived NO in this conduit artery. In contrast, relaxation to NO-donors appears to be mediated entirely via cyclic GMP-dependent mechanisms.

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