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Evidence that potassium channels make a major contribution to SIN-1-evoked relaxation of rat isolated mesenteric artery

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1 The NO donor 3-morpholino-sydnonimine (SIN-1; 0.01 – 10 μM) evoked concentration-dependent relaxation of rat isolated mesenteric arteries pre-constricted with phenylephrine (1 – 3 μM). The relaxation to SIN-1 was not significantly different between endothelium-intact or denuded arterial segments or segments in which basal nitric oxide (NO) synthesis was inhibited ($n=8$; $P>0.05$). In contrast, the membrane permeable analogue of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), 8-Br-cyclic GMP (0.01 – 1 mM), was much less effective in relaxing intact than denuded arterial segments or intact arterial segments pre-incubated with NO synthase blockers ($n=4$; $P<0.01$).

2 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM; 10 min) alone, did not alter SIN-1-evoked relaxation in any tissues ($n=5$; $P>0.05$). However, in parallel experiments, ODQ almost completely inhibited both basal and SIN-1-stimulated production of cyclic GMP in both the presence and absence of NO synthase blockers ($n=6$; $P<0.01$) indicating that full relaxation to SIN-1 can be achieved in the absence of an increase in cyclic GMP.

3 Exposure of endothelium-intact arterial segments to the potassium channel blocker charybdotoxin (50 nM; 10 min), significantly inhibited SIN-1-evoked relaxation, reducing the maximum response by around 90% ($n=5$; $P<0.01$). In contrast, in arterial segments in which either the endothelial cell layer had been removed or basal NO synthesis inhibited, relaxation to SIN-1 was not reduced in the presence of charybdotoxin ($n=6$; $P>0.05$). However, in the presence of NO synthase blockers and L-arginine (300 μM) together, charybdotoxin did significantly inhibit SIN-1-evoked relaxation to a similar extent as intact tissues (maximum response reduced by around 80%; $n=4$; $P<0.01$).

4 Pre-incubation with apamin (30 nM; 10 min) or glibenclamide (10 μM; 10 min) did not alter SIN-1-evoked relaxation of phenylephrine-induced tone in any tissues ($n=4$ and $n=6$, respectively; $P>0.05$). However, in the presence of either ODQ and apamin, or ODQ and glibenclamide, SIN-1-evoked relaxation was significantly attenuated in intact arterial segments and segments in which NO synthesis was blocked.

5 Exposure of intact arterial segments to charybdotoxin and apamin, in the presence of NO synthase blockers, also significantly inhibited SIN-1-evoked relaxation, reducing the maximum response by around 80% ($n=4$; $P<0.01$).

6 Addition of superoxide dismutase (SOD; 30 u ml^{-1}), potentiated relaxations to SIN-1 in all tissues, but did not alter the effects of charybdotoxin and ODQ on SIN-1-evoked relaxation.

7 These data show that although relaxation to the NO-donor SIN-1 is not significantly different between endothelium-intact and denuded arterial segments, the mechanisms which mediate SIN-1-evoked relaxation in the rat isolated mesenteric artery appear to be modulated by the basal release of endothelium-derived NO. In the presence of an intact endothelial cell layer, the major mechanism for SIN-1-evoked relaxation appears to be the activation of charybdotoxin-sensitive potassium channels. In contrast, when basal NO synthesis is inhibited, SIN-1 appears to cause full relaxation by both the activation of a charybdotoxin-sensitive pathway and the stimulation of soluble guanylyl cyclase.

Keywords: Nitric oxide; endothelium; cyclic GMP; vascular smooth muscle

Introduction

Nitric oxide (NO) is generally thought to cause smooth muscle relaxation by stimulation of soluble guanylyl cyclase leading to an increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP), with the subsequent activation of cyclic GMP-dependent kinases leading to relaxation (for review see Ignarro, 1990). Recent studies have shown that NO can also cause hyperpolarization of smooth muscle cells (Garland & McPherson, 1992; Plane *et al.*, 1994). In rat mesenteric arteries, NO-evoked hyperpolarization of the resting membrane potential is sensitive to the inhibitor of ATP-sensitive potassium channels, glibenclamide (Garland & McPherson, 1992), whereas in rabbit mesenteric arteries this response is apparently inhibited by apamin, which blocks small conductance calcium-sensitive potassium channels (Murphy & Brayden, 1995). Furthermore, NO can stimulate charybdotoxin-sensitive potassium channels, either directly (Bolotina *et al.*, 1994) or via a cyclic GMP-dependent mechanism (George & Shibata, 1995). However, the contribution which potassium channels make to NO-evoked vasodilatation is unclear.

In the present study, the extent to which potassium channels contribute to NO-induced smooth muscle relaxation was assessed with the NO donor 3-morpholino-syndnonimine (SIN-1) in rat small mesenteric arteries. Some of these results have been presented to the British Pharmacological Society (Plane *et al.*, 1996).

Methods

Male Wistar rats (250 – 350 g) were stunned and killed by cervical dislocation. The mesentery was removed and placed in

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Krebs buffer. Segments (2 mm in length; D_{100} 310±10 μM; $n=30$) of third order branches of the superior mesenteric artery were removed and mounted in a Mulvany-Halpern myograph (model 400A, J.P. Trading, Denmark) under a normalised tension as previously described (Garland & McPherson, 1992). Tissues were maintained at 37°C in Krebs buffer containing indomethacin (2.8 μM) bubbled with 95% O_2/5% CO_2. In some experiments the endothelium was removed by rubbing the intima with a hair.

After an initial equilibration period of 60 min, the integrity of the endothelium was assessed by pre-contracting the tissues with phenylephrine (1–3 μM) and then adding acetylcholine (1 μM). Tissues in which the acetylcholine reversed phenylephrine-induced tone by more than 90% were designated as endothelium intact and tissues in which acetylcholine caused less than 10% relaxation were designated as denuded. In some experiments, NO synthesis in intact arterial segments was inhibited by addition of the NO synthase blockers L-N^G-nitro-arginine (L-NOARG) and L-N^G-nitro-arginine methyl ester (L-NAME; both 100 μM) 30 min before the first addition of SIN-1 and then throughout the rest of the experiment.

In a parallel study, increases in tissue cyclic GMP levels in 2 m long segments of rat mesenteric artery were measured by radioimmunoassay (Amersham International) in the presence of isobutylmethylxanthine (IBMX) as previously described (Miller *et al.*, 1994). Tisuses were stimulated with phenylephrine (3 μM) and SIN-1 (10 μM) together for 15 min in the presence and absence of the various inhibitors.

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.

Drugs used were from Sigma except for charybdotoxin (CalBiochem), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris) and SIN-1 (Tocris).

Analysis of data

In all experiments, relaxations are expressed as a percentage decrease in the induced level of tone. All data are expressed as mean±s.e.mean. The significance of differences between mean values was calculated by Student's *t* test, with rejection of the null hypothesis at the 5% level.

Results

Influence of the endothelium on SIN-1- and 8-Br-cyclic GMP-evoked relaxation

SIN-1 (0.01–10 μM) elicited concentration-dependent relaxation of phenylephrine (1–3 μM)-stimulated arterial segments, which was not significantly different between endothelium-intact segments, denuded segments and intact segments in which basal NO synthesis had been inhibited ($n=8$; $P>0.05$). Concentration-response curves for SIN-1-evoked relaxations in the three tissue groups are shown in Figure 1a.

The membrane permeable analogue of cyclic GMP, 8-Br-cyclic GMP (0.01–1 mM) caused almost full relaxation of denuded arterial segments and endothelium intact segments pre-incubated with NO synthase inhibitors, reducing the phenylephrine-evoked contraction by 90.6±5.7% and 94.5±1.6%, respectively ($n=4$). In contrast, in endothelium-intact segments, 8-Br-cyclic GMP only stimulated a maximum relaxation of 28.7±4.3% ($n=4$; $P<0.01$). Concentration-response curves for 8-Br-cyclic GMP-evoked relaxations in intact arterial segments and arterial segments exposed to NO synthase blockers are shown in Figure 1b.

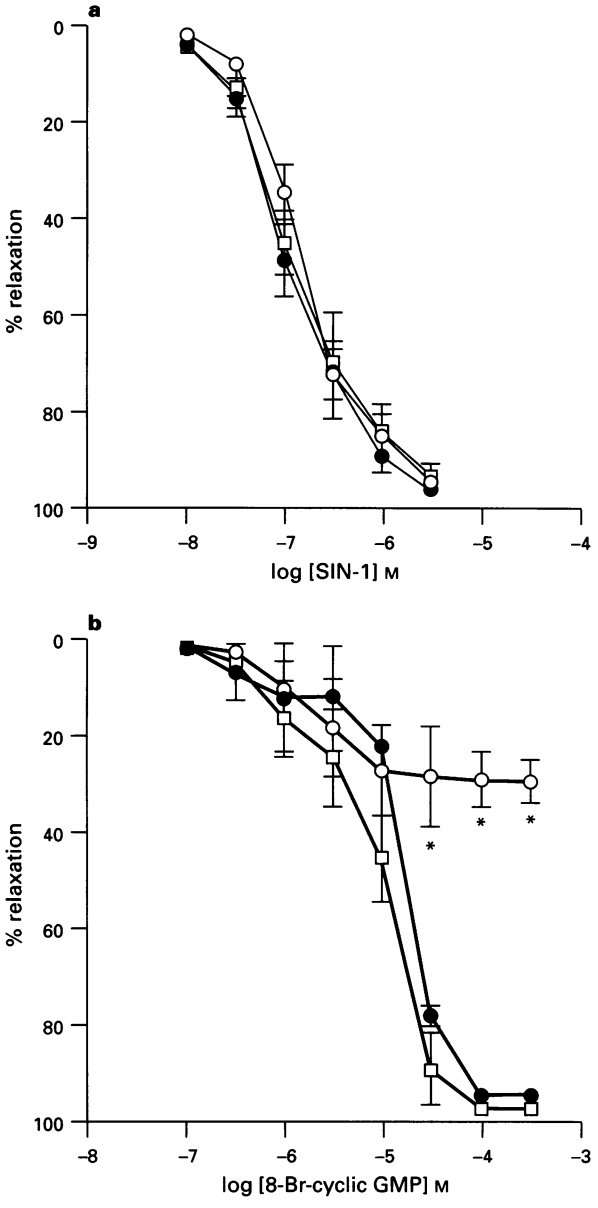

Figure 1 Mean concentration-response curves for (a) SIN-1 and (b) 8-Br-cyclic GMP-evoked relaxation of rat isolated mesenteric arteries. (○) Intact arterial segments, (□) denuded segments and (●) intact segments exposed to NO synthase inhibitors. All values are means from (a) 8 and (b) 4 experiments with s.e. means shown by vertical lines. *$P<0.01$ compared to endothelium-denuded tissues.

Role of cyclic GMP in SIN-1-evoked relaxation

The inhibitor of soluble guanylyl cyclase, ODQ (10 μM; 10 min; Garthwaite *et al.*, 1995), did not inhibit the relaxation to SIN-1 in either denuded arterial segments, or in intact segments in the presence of NO synthase inhibitors ($n=5$; $P>0.05$). In intact tissues, SIN-1-evoked relaxations were in fact potentiated in the presence of ODQ ($n=5$; $P>0.05$). Concentration-response curves for SIN-1-evoked relaxation of intact, denuded and intact segments exposed to NO synthase inhibitors, in the presence and absence of ODQ are shown in Figure 2.

In parallel experiments, SIN-1 (10 μM) stimulated a three fold increase in cyclic GMP levels in endothelium-intact tissues exposed to phenylephrine (Figure 3; $n=6$; $P<0.01$). In the presence of NO synthase inhibitors, the basal level of cyclic GMP production was significantly reduced ($n=6$; $P<0.01$), but the rate of cyclic GMP production stimulated by SIN-1 in

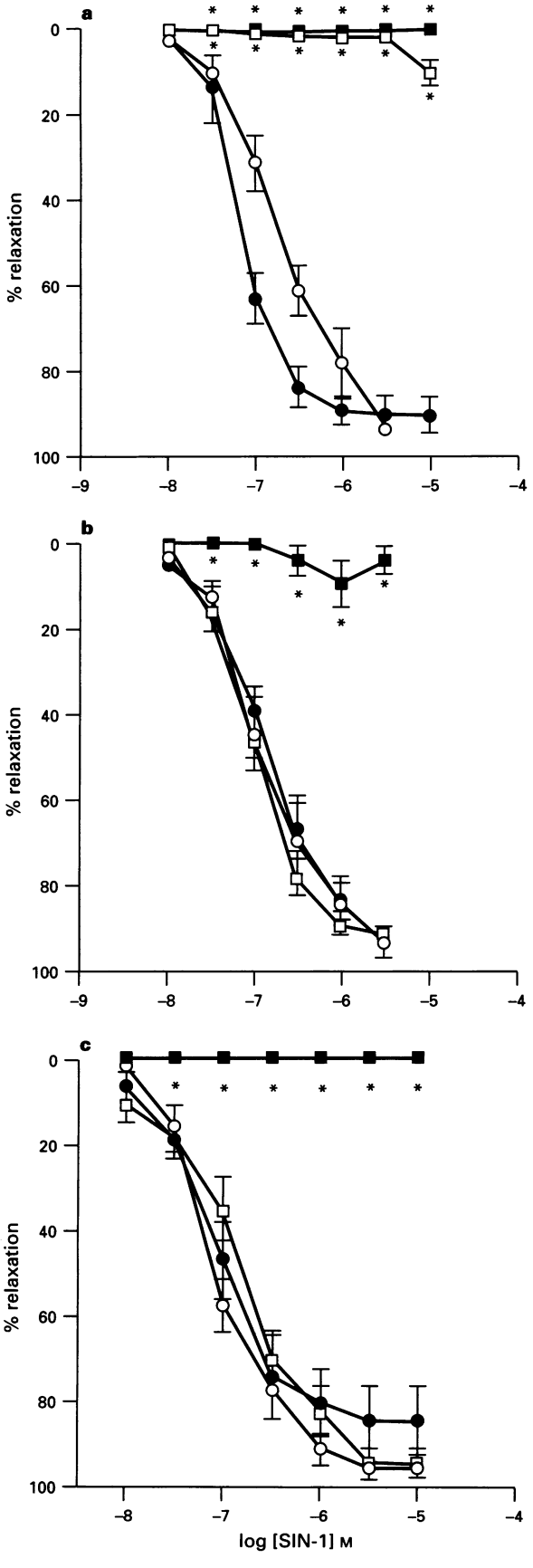

Figure 2 Mean concentration-response curves for SIN-1-evoked relaxation of (a) intact arterial segments, (b) denuded segments and (c) tissues exposed to NO synthase blockers. Experiments were carried out in the absence (○) or presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM; ●), charybdotoxin (50 nM; □) or ODQ and charybdotoxin together (■). All values are means from 5 experiments with s.e. means shown by vertical lines. *$P<0.01$ compared to control values.

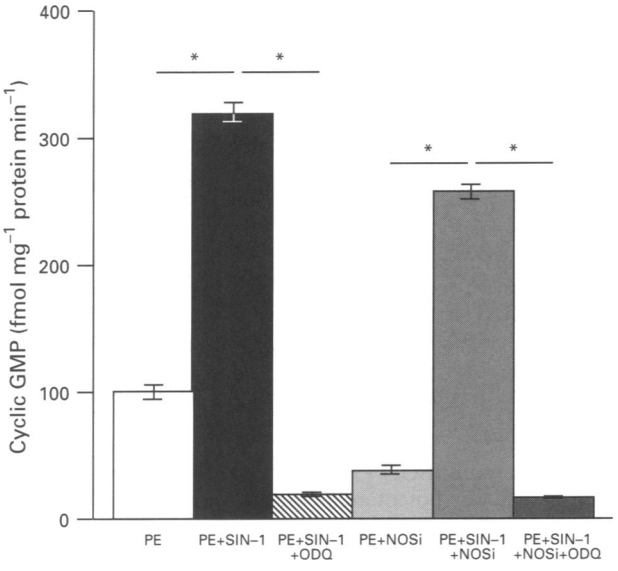

Figure 3 Effect of SIN-1 (100 μM) on the rate of cyclic GMP formation in endothelium-intact rat mesenteric arteries in the presence and absence of ODQ (100 μM) and/or the NO synthase inhibitors (NOSi), L-NAME and L-NOARG (both 100 μM). All values are means from 6 experiments with s.e. means shown by vertical bars. *$P<0.01$.

these tissues was of a similar magnitude to that observed in the absence of the NO synthase inhibitors ($n=6$). ODQ almost completely inhibited both basal and SIN-1-stimulated production of cyclic GMP in both the absence and presence of NO synthase inhibitors ($n=6$; $P<0.01$; Figure 3).

Role of potassium channels in SIN-1-evoked relaxation

In tissues with an intact endothelial cell layer, SIN-1-evoked relaxation was almost completely inhibited by charybdotoxin (50 nM; 10 min), with the maximum response being reduced to $9.8\pm2.7\%$ ($n=5$; $P<0.01$). In contrast, in denuded segments and in arterial segments pre-incubated with NO synthase blockers, exposure to charybdotoxin alone did not significantly inhibit SIN-1-evoked relaxation (maximum relaxation $94.8\pm3.5\%$ and $91.8\pm2.3\%$, respectively, $n=6$; $P>0.05$). However, exposure to ODQ and charybdotoxin together abolished the SIN-1-evoked relaxation ($n=6$; $P<0.01$). Concentration-response curves for SIN-1-evoked relaxation in endothelium intact artery segments, denuded segments and segments exposed to NO synthase blockers, in the presence and absence of charybdotoxin and ODQ are shown in Figure 2.

Pre-incubation of endothelium-intact tissues with the NO synthase blockers and L-arginine (300 μM; 30 min) together had no effect on SIN-1-evoked responses ($n=4$; $P>0.05$). However, under these conditions, exposure to charybdotoxin significantly inhibited SIN-1-evoked relaxations and reduced the maximum response to a similar extent to that observed in endothelium-intact tissues in the absence of NO synthase inhibitors (maximum response $20.4\pm8.2\%$; $n=4$; $P<0.01$).

In intact tissues previously exposed to the NO synthase inhibitors and charybdotoxin, exposure to L-arginine for 60 min, in the continued presence of these agents, caused a significant inhibition of SIN-1-evoked relaxation and reduced the maximum response to $59.6\pm7.4\%$ ($n=4$; $P<0.01$).

Exposure to either apamin (30 nM; 10 min), an inhibitor of small conductance potassium channels, or glibenclamide (10 μM; 10 min), an inhibitor of ATP-sensitive potassium channels, did not significantly alter SIN-1-evoked responses in any tissue ($n=4$ and $n=6$, respectively; $P>0.05$). However, in the combined presence of either ODQ and apamin, or ODQ

and glibenclamide, SIN-1-evoked relaxation of either endothelium-intact segments or arterial segments exposed to NO synthase blockers, was attenuated. In intact tissues in the presence and absence of NO synthase blockers, ODQ and apamin combined reduced the maximum response to 69.4±4.2% and 77.6±4.9% ($n=4$; $P<0.01$), respectively (Figure 4a and b). In the presence of glibenclamide and ODQ, the maximal relaxation of intact tissues in the presence and absence of NO synthase blockers was reduced to 84.9±2.6% and 64.0±6.0% ($n=6$; $P<0.01$), respectively (Figure 4c and d). Exposure of tissues in which NO synthesis was blocked to a combination of apamin and charybdotoxin also significantly inhibited SIN-1-evoked responses reducing the maximum response to 20.4±7.6% ($n=4$; $P<0.01$; Figure 4b).

The effect of superoxide dismutase (SOD) on SIN-1-evoked relaxation

In the presence of SOD (30 u ml^{-1}), SIN-1-evoked relaxation of all tissues was potentiated (Figure 5). However, in the presence of SOD the effects of charybdotoxin and ODQ on SIN-1-evoked relaxation of intact arterial segments and segments exposed to NO synthase inhibitors were not significantly different from those observed in the absence of this free radical scavenger and described above.

Discussion

These data provide the first functional evidence to suggest that activation of a charybdotoxin-sensitive pathway makes a major contribution to NO-induced relaxation of vascular smooth cells, independent of any increase in cyclic GMP. Further, they indicate that the mechanisms mediating NO-evoked relaxation are modulated by the basal release of endothelium-derived NO.

In this study, the NO donor SIN evoked relaxation of phenylephrine-induced tone which was not significantly different in endothelium-intact segments of mesenteric artery, denuded artery segments and segments in which basal NO

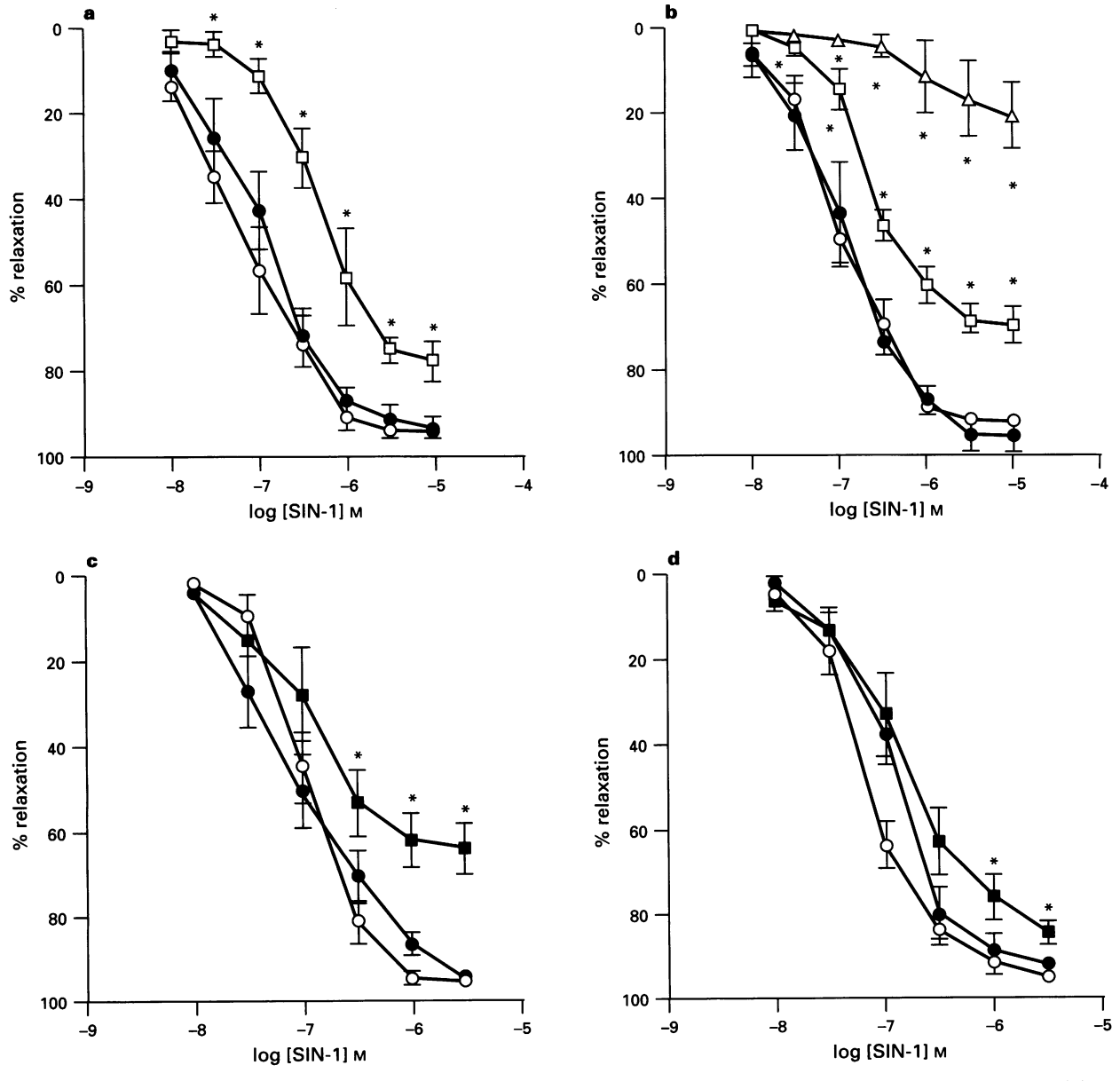

Figure 4 Effect of apamin (30 nM; a and b) and glibenclamide (10 μM; c and d) on SIN-1-evoked relaxation of intact arterial segments in the presence (b and d) and absence (a and c) of NO synthase inhibitors. Experiments were carried out in the absence (○) and presence (●) of apamin or glibenclamide alone, or (□) ODQ (10 μM) and either apamin or glibenclamide, or apamin and charybdotoxin (△). All values are means from 4 experiments with s.e. means shown by vertical lines. *$P<0.01$.

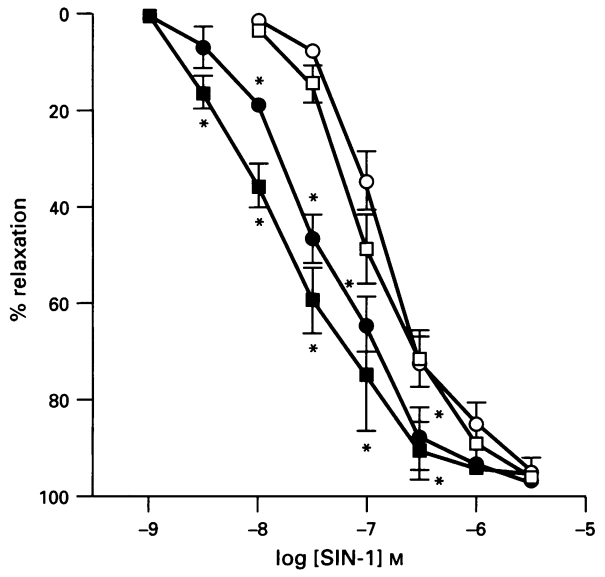

Figure 5 Mean concentration-response curves for SIN-1-evoked relaxation of intact arterial segments in the absence (○) or presence (□) of NO synthase blockers and SOD (30 u ml^{-1}; ●; ■). All values are means from 4 experiments with s.e. means shown by vertical lines. *$P<0.01$ compared to control values in the absence of SOD.

synthesis was inhibited by exposure to NO synthase blockers. This is in contrast to several previous studies of larger, conduit arteries in which endothelium removal was shown to potentiate relaxation to NO donors (Busse *et al.*, 1990). However, although the presence of the endothelium did not alter the sensitivity of the smooth muscle to SIN-1, the mechanisms mediating SIN-1-evoked relaxation did appear to be modulated by the basal release of NO.

Recent studies have shown that NO can stimulate charybdotoxin-sensitive potassium channels, either directly (Bolotina *et al.*, 1994) or via a cyclic GMP-dependent mechanism (George & Shibata, 1995), and that cyclic GMP itself can directly activate potassium channels (Robertson *et al.*, 1993). In mesenteric resistance arteries, SIN-1-evoked relaxation of endothelium-intact arterial segments was almost completely inhibited by charybdotoxin but was unaffected by the soluble guanylyl cyclase inhibitor ODQ, at a concentration which abolished SIN-1-induced increases in cyclic GMP. This indicates that in the presence of an intact endothelial cell layer, SIN-1-evoked relaxation can be fully accounted for by the activation of a charybdotoxin-sensitive pathway with little or no contribution from pathways activated by increased levels of cyclic GMP.

In contrast, in arterial segments in which basal NO production was blocked either with NO synthase inhibitors or by endothelial cell removal, neither charybdotoxin nor ODQ alone were effective in reducing smooth muscle relaxation. Both agents together were required to inhibit the relaxation to SIN-1. These observations indicate that in the absence of basal NO production, both an ODQ-sensitive pathway and a charybdotoxin-sensitive pathway can mediate complete smooth muscle relaxation. However, in the presence of basal NO production by the endothelium, the relaxation mediated via an ODQ-sensitive pathway appears to be supressed.

Previous work has indicated that basal NO can modulate the metabolism or actions of cyclic GMP in vascular smooth muscle, probably at a site distal to the activation of soluble guanylyl cyclase (Busse *et al.*, 1990). This proposal is supported by two observations in the present study. First, SIN-1 was able to elevate cyclic GMP in intact arterial segments even though the elevation appeared not to contribute to the observed relaxation. Second, 8-Br-cyclic GMP was less effective in stimulating relaxation in endothelium-intact arterial segments than in segments in which either the basal production of NO was inhibited or in which the endothelium had been removed. Together, these observations indicate that endothelium-dependent inhibition of the actions of cyclic GMP may play an important role in maintaining tone in resistance artery smooth muscle cells.

A modulator role for basal NO production was confirmed by experiments in which NO synthase inhibition was reversed by the addition of exogenous L-arginine. In tissues pre-incubated with L-arginine and NO synthase inhibitors together, SIN-1-evoked relaxations showed a similar sensitivity to charybdotoxin as intact arterial segments, presumably because the L-arginine prevented the total inhibition of NO synthase. Furthermore, in intact tissues in which NO synthesis was inhibited and charybdotoxin had little effect on SIN-1-evoked relaxation, the addition of L-arginine for 60 min partially restored the sensitivity of SIN-1-evoked relaxation to block with charybdotoxin. These results demonstrate that the differences in the sensitivity of SIN-1-evoked relaxation to charybdotoxin and ODQ in intact and denuded tissues reflect basal NO synthesis, and that the effects observed with the NO synthase blockers were due to inhibition of NO synthesis rather than a non-specific action of these inhibitors.

How basal NO production inhibits the ability of cyclic GMP to cause relaxation is not clear at present. One possibility is that high basal NO release may lead to an up-regulation of cyclic GMP-dependent phophodiesterase, which would accelerate the breakdown of cyclic GMP and thus reduce its effect. Although SIN-1 was able to stimulate increases in cyclic GMP, the assay for cyclic GMP accumulation was performed in the presence of the phophodiesterase inhibitor (IBMX) making it impossible to assess this suggestion.

Previous studies have indicated that both ATP-sensitive potassium channels (Garland & McPherson, 1992) and small conductance calcium-dependent potassium channels (Murphy & Brayden, 1995) may mediate NO-evoked hyperpolarization of the resting membrane potential in mesenteric arteries. In the present study, neither glibenclamide nor apamin individually altered SIN-1-evoked relaxation, indicating that in pre-contracted arteries the activation of either apamin- or glibenclamide-sensitive potassium channels does not make a significant contribute to NO-evoked relaxation under normal conditions. However, in the presence of ODQ, both inhibitors did individually reduce the maximum relaxation to SIN-1, indicating that activation of the respective potassium channels contributes to NO-evoked relaxation, but only when cyclic GMP production is blocked.

In tissues in which NO synthesis was blocked, exposure to a combination of apamin and charybdotoxin significantly inhibited SIN-1-induced relaxation, whereas neither of these agents alone had any significant effect. These results indicate that either SIN-1 activates both apamin and charybdotoxin-sensitive pathways, both of which can mediate full relaxation, or that there is one pathway which is sensitive to both of these inhibitors. The latter suggestion has recently been proposed by Zygmunt & Hogestatt (1996), to explain the finding that, in rat hepatic artery, endothelium-dependent relaxation to acetylcholine was unaffected by either apamin or charybdotoxin alone, but inhibited by the two blockers in combination.

In addition to releasing NO, SIN-1 also releases superoxide anions which could potentially react with NO to form peroxynitrite. To ensure that the cyclic GMP-independent component of the relaxation to SIN-1 was not due to peroxynitrite, experiments were also carried out in the presence of SOD. SOD significantly potentiated relaxations to SIN-1 in all tissues, signifying that NO released from SIN-1 was being broken down by superoxide anion in our experiments. However, the presence of SOD did not alter the sensitivity of SIN-1-evoked relaxations to either charybdotoxin or ODQ in intact arterial segments or segments in which basal NO synthesis was inhibited. This indicates that metabolites of NO do not contribute to the observed cyclic GMP-independent responses.

In conclusion, these data indicate the mechanisms which mediate the dilator actions of the NO donor SIN-1 are modulated by the basal production of endothelium-derived NO in rat isolated mesenteric resistance arteries. Furthermore, in arteries with an intact endothelial cell layer, as occurs *in vivo*, the activation of charybdotoxin-sensitive potassium channels appears to be more important than the activation of soluble guanylyl cyclase in mediating the dilator action of the NO donor SIN-1.

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