British Journal of Pharmacology (1997) 122, 117–125

Central role of intracellular calcium stores in acute flow- and agonist-evoked endothelial nitric oxide release

[1]Iain R. Hutcheson & Tudor M. Griffith

Cardiovascular Sciences Research Group, Department of Diagnostic Radiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN

1 We have used a cascade bioassay system and isolated arterial ring preparations to investigate the contribution of Ca^{2+} release from endothelial intracellular stores to nitric oxide (NO) production evoked by increases in shear stress and by acetylcholine in rabbit aorta.

2 Experiments were performed before and following incubation with either the endoplasmic reticulum Ca^{2+}-ATPase inhibitors cyclopiazonic acid (CPA, 10 μM) and thapsigargin (TSG, 1 μM) or ryanodine (30, 100 μM) which binds to a specific endoplasmic reticulum Ca^{2+}-release channel.

3 In cascade bioassay all three agents induced relaxations of the recipient ring (CPA, $24.4 \pm 3.8\%$; TSG, $51.5 \pm 10.6\%$; ryanodine, $17.4 \pm 1.6\%$) which were significantly attenuated by preincubation of the donor with 100 μM N^G-nitro-L-arginine methyl ester (L-NAME). However, in isolated rings, only CPA and TSG induced L-NAME-sensitive relaxations (CPA $52.7 \pm 6.5\%$; TSG $61.3 \pm 7\%$).

4 Addition of superoxide dismutase (SOD) to the donor perfusate evoked relaxations of the recipient ring in cascade bioassay ($13.3 \pm 1.4\%$, $n=22$). Prior administration of SOD attenuated relaxations to TSG ($23.2 \pm 3.8\%$, $n=4$) and ryanodine ($1.7 \pm 0.8\%$, $n=4$), and pre-incubation with TSG and ryanodine blunted SOD-induced responses ($4 \pm 1.5\%$, $n=4$ and $8.9 \pm 1.1\%$, $n=4$, respectively). By contrast, no interaction was observed between the relaxations evoked by SOD and CPA. In isolated rings, SOD exerted no direct relaxant action and did not modulate relaxations to CPA, TSG or ryanodine.

5 In cascade bioassay studies time-averaged shear stress was manipulated with dextran (1–4% w/v, 80000 MW) to increase perfusate viscosity. NO-dependent relaxation of the recipient ring induced by increased perfusate viscosity was significantly attenuated by CPA ($P<0.01$; $n=6$) and TSG ($P<0.05$; $n=7$), but not by ryanodine ($n=6$).

6 Endothelium-dependent relaxations to acetylcholine (0.1–30 μM) in cascade bioassay and in isolated aortic ring preparations were markedly attenuated by pretreatment with CPA and TSG, but were unaffected by ryanodine. Ryanodine and CPA caused only a small attenuation of endothelium-independent relaxations to sodium nitroprusside (0.001–10 μM), whereas TSG had no effect.

7 We conclude that release of Ca^{2+} from CPA- and TSG-sensitive endothelial stores is necessary for NO release evoked by acute flow changes and agonists in rabbit abdominal aorta. Ca^{2+}-induced Ca^{2+} release via the ryanodine-sensitive release channel plays no direct role in these responses. Free radical interactions may complicate the interpretation of findings in cascade bioassay compared with isolated ring preparations.

Keywords: Rabbit aorta; shear stress; acetylcholine; ryanodine; cyclopiazonic acid; thapsigargin; superoxide dismutase (SOD); nitric oxide (NO)

Introduction

The endothelial intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response to agonists is biphasic consisting of an initial transient rise, which is independent of extracellular Ca^{2+}, followed by a sustained elevation mediated by Ca^{2+} influx (see Adams *et al*., 1993 for review). The intracellular Ca^{2+} store contributes to both phases of this response. The initial Ca^{2+} transient reflects release of Ca^{2+} from inositol trisphosphate (IP_3)-sensitive stores (Derian & Moskowitz, 1986; Lambert *et al*., 1986; Freay *et al*., 1989) and recent findings have suggested that depletion of internal stores can then act as the signal for Ca^{2+} influx through activation of the capacitative Ca^{2+} entry pathway (Schilling *et al*., 1992; Dolor *et al*., 1992; Zheng *et al*., 1994; Moritoki *et al*., 1994; Pasyk *et al*., 1995). A key role for the intracellular Ca^{2+} store in agonist-evoked NO release has consequently been proposed since the endothelial $[Ca^{2+}]_i$ is a crucial determinant for the production of nitric oxide (NO), as the constitutive NO synthase is Ca^{2+}/calmodulin-dependent (Moncada *et al*., 1991). This has been confirmed by the finding that there is a close correlation between intracellular Ca^{2+} mobilization and NO release in bovine aortic endothelial cells stimulated with bradykinin (Blatter *et al*., 1995). Furthermore, thapsigargin, an inhibitor of the endothelial endoplasmic reticulum Ca^{2+}-ATPase, which depletes the IP_3-sensitive intracellular store, abolishes agonist-evoked NO release from bovine aortic endothelial cells and intact segments of rabbit aorta and femoral artery (Macarthur *et al*., 1993; Amerini *et al*., 1996).

Flow, like agonists, can increase $[Ca^{2+}]_i$ biphasically in cultured endothelial cells, the transient rise occurring in the absence of extracellular Ca^{2+} again implicating the involvement of intracellular Ca^{2+} stores in this response (Geiger *et al*., 1992; Helminger *et al*., 1995; 1996; Kanai *et al*., 1995). This is reinforced by findings that shear stress can elevate IP_3 levels in bovine aortic and human umbilical vein endothelial cells (Nollert *et al*., 1990; Bhagyalakshmi *et al*., 1992; Prasad *et al*., 1993) and that thapsigargin abolishes shear stress-induced $[Ca^{2+}]_i$ oscillations in bovine cultured aortic endothelial cells (Helminger *et al*., 1996). However, the importance of this intracellular signalling mechanism in the subsequent release of NO in response to shear stress remains unclear. Indeed, simultaneous measurement of $[Ca^{2+}]_i$ and NO production in bovine aortic endothelial cells in response to flow has demonstrated that the initial $[Ca^{2+}]_i$ transient preceding NO release is entirely dependent on the presence of extracellular Ca^{2+} (Kanai *et al*., 1995).

[1] Author for correspondence.

Recently, it has been demonstrated that ryanodine-sensitive Ca^{2+} channels are present in vascular and endocardial endothelium (Lesh *et al*., 1993; Ziegelstein *et al*., 1994; Wang *et al*., 1995). Ryanodine binds to the sarcoplasmic reticulum Ca^{2+} release channel in skeletal, cardiac and smooth muscle, keeping it in an open, subconductance state at low concentrations but blocking it at higher concentrations. In a variety of cultured endothelial cells ryanodine induces a slow increase in $[Ca^{2+}]_i$ (Ziegelstein *et al*., 1994) and, in rabbit fresh aortic endothelial cells, it attenuates acetylcholine-induced $[Ca^{2+}]_i$ transients, suggesting that a Ca^{2+}-induced Ca^{2+} release mechanism may also contribute to the agonist-evoked $[Ca^{2+}]_i$ response (Wang *et al*., 1995). An involvement in NO synthesis has also been suggested from the finding that caffeine can stimulate NO synthesis by release of Ca^{2+} from a ryanodine-sensitive channel in rat aortic endothelium (Hatano *et al*., 1995). However, there is as yet no definitive evidence for the involvement of this mechanism in flow- or agonist-induced NO release.

It has been shown that NO release in response to flow is biphasic consisting of an initial transient component (15–20 min) that is Ca^{2+}-dependent followed by a sustained Ca^{2+}-independent release (Kuchan & Frangos, 1994; Ayajiki *et al*., 1996). In the present study, we have investigated the acute (0–15 min), Ca^{2+}-dependent phase of flow- and agonist-induced NO release by use of a cascade bioassay system and endothelium-intact arterial ring preparations to clarify the role of intracellular Ca^{2+} stores and investigate the potential involvement of the ryanodine receptor. Cyclopiazonic acid, thapsigargin and ryanodine were used to manipulate intracellular Ca^{2+} store function. A previous study suggested that depletion of stores with thapsigargin has no effect on flow-related NO production in either cultured endothelial cells or intact arteries at levels of arterial shear stress below those found under physiological conditions (Macarthur *et al*., 1993). In the present study, therefore, experiments were designed to produce levels that approximate those found *in vivo*.

Methods

Experiments were performed with abdominal and thoracic aortae from male New Zealand white rabbits (2.5 kg) which had been killed by injection of sodium pentobarbitone (120 mg kg^{-1}; i.v.). The tissues were transferred to cold (4°C) gassed (95% O_2, 5% CO_2, pH 7.4) Holman's solution of the following composition (mM): NaCl 120, KCl 5, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11, sucrose 10, containing indomethacin (10 μM). N^G-nitro-L-arginine methyl ester (L-NAME) was used as a specific inhibitor of NO formation from L-arginine in both cascade bioassay and organ bath experiments.

Cascade bioassay studies

The bioassay system used to quantify NO release in response to changes in time-averaged shear stress has been described previously (Hutcheson & Griffith, 1996). Briefly, a 4 cm segment of 'donor' endothelium-intact rabbit abdominal aorta was cannulated, positioned horizontally in an organ bath and perfused with gassed Holman's buffer (37°C) at a constant flow rate of 9 ml min^{-1}. Perfusion pressure was monitored continuously by a pressure transducer (SensoNor 840) positioned immediately proximal to the infusion cannula. NO release was monitored by a ring of endothelium-denuded rabbit thoracic aorta (the recipient) preconstricted with phenylephrine (300 nM) which was positioned directly below the organ bath outlet (transit time from donor to recipient $\sim$2 s). The presence of donor endothelium was confirmed histologically by *en face* silver staining at the end of each experiment.

To study changes in viscosity and thus time-averaged shear stress at a constant mean flow rate of 9 ml min^{-1}, dextran (80,000 MW; 1–4% w/v) was added to the perfusate. Perfusate viscosity was measured with a clinical viscometer (Luckham). Responses to agonist stimulation were also assessed by construction of cumulative concentration-response curves to acetylcholine added to the perfusate. For these experiments atropine (3 μM) was superfused over the recipient ring to eliminate any direct vasoconstrictor effect. Relaxations to acetylcholine were also studied in the absence and presence of dextran (1% and 2%) in the perfusate to assess the effect of viscosity changes on agonist-evoked responses.

To investigate the role played by the intracellular Ca^{2+} stores the experiments were repeated either following a 20 min incubation of donor aorta and recipient ring with TSG (1 μM) and ryanodine (30, 100 μM) which act irreversibly (Macarthur *et al*., 1993; Ziegelstein *et al*., 1994; Rusko *et al*., 1995) or in the continuous presence of CPA (10 μM).

Isolated ring preparations

Isolated endothelium-intact rabbit abdominal aortic rings were suspended in organ baths containing gassed Holman's solution at 37°C and precontracted with a submaximal concentration of phenylephrine (300 mM). Cumulative concentration-response curves to acetylcholine were then constructed before and following addition of CPA (10 μM), TSG (1 μM) and ryanodine (30, 100 μM) to the organ bath. These agents were added 1 h before the administration of acetylcholine. The effect of increasing concentrations of dextran (1–4%) were also assessed in endothelium-intact and -denuded rings of abdominal aorta. Relaxations to sodium nitroprusside (SNP) were assessed in similar fashion, with endothelium-denuded thoracic aortic rings, to determine any effects of these agents on smooth muscle function.

Drugs

Acetylcholine, phenylephrine, dextran 80, dimethylsulphoxide (DMSO), indomethacin, atropine, cyclopiazonic acid, thapsigargin, L-arginine, superoxide dismutase and N^G-nitro-L-arginine methyl ester were obtained from Sigma Limited (Poole, U.K.). Ryanodine was obtained from Semat Technical (UK) Ltd (St. Albans, U.K.). All drugs were dissolved in Holman's buffer with the exception of CPA and TSG (DMSO), ryanodine (distilled water), and indomethacin (5% w/v $NaHCO_3$ in distilled water).

Statistics

All data are given as mean$\pm$s.e.mean, where n denotes the number of animals studied for each data point. Statistical analysis for viscosity studies was assessed by repeated measures ANOVA followed by the Bonferroni multiple comparisons test and for acetylcholine and SNP studies the Student's t test for paired data was used. $P<0.05$ was considered as significant.

Results

Cascade bioassay studies

Effects on basal NO release Addition of CPA (10 μM) or TSG (1 μM) to the donor perfusate caused transient relaxations of the recipient ring of 24.4$\pm$3.8% ($n=7$) and 51.1$\pm$10.6% ($n=7$), respectively, with tone returning to 94$\pm$6% and 91$\pm$5% of its original value after 75 min (Figure 1). This slow rise in tension was not due to depletion of the endothelial L-arginine pool as subsequent administration of exogenous L-arginine (10 mM) did not restore the dilator responses ($n=4$, not shown). In these studies the concentration of DMSO in the perfusate was 0.01% which was found, in separate experiments, to cause an L-NAME-insensitive relaxation of the recipient ring which peaked at 8.7$\pm$0.5% after 3 min but declined to 3.7$\pm$0.2% after 60 min ($n=7$). Ryanodine (30 μM) also induced relaxations of the recipient ring (17.4$\pm$1.6%,

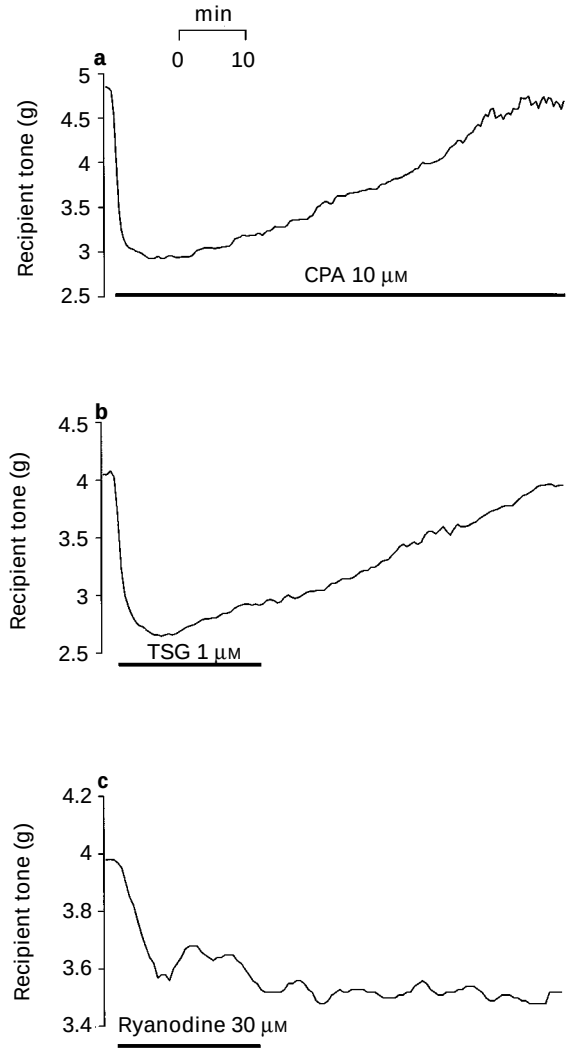

Figure 1 Representative traces of relaxations of isolated rings of rabbit abdominal aorta, used as donor tissue in cascade bioassay, when cyclopiazonic acid (CPA; a), thapsigargin (TSG; b) and ryanodine (c) were included in the buffer. Relaxations to CPA and TSG generally returned to baseline within 30–60 min, whereas ryanodine-induced relaxations were sustained.

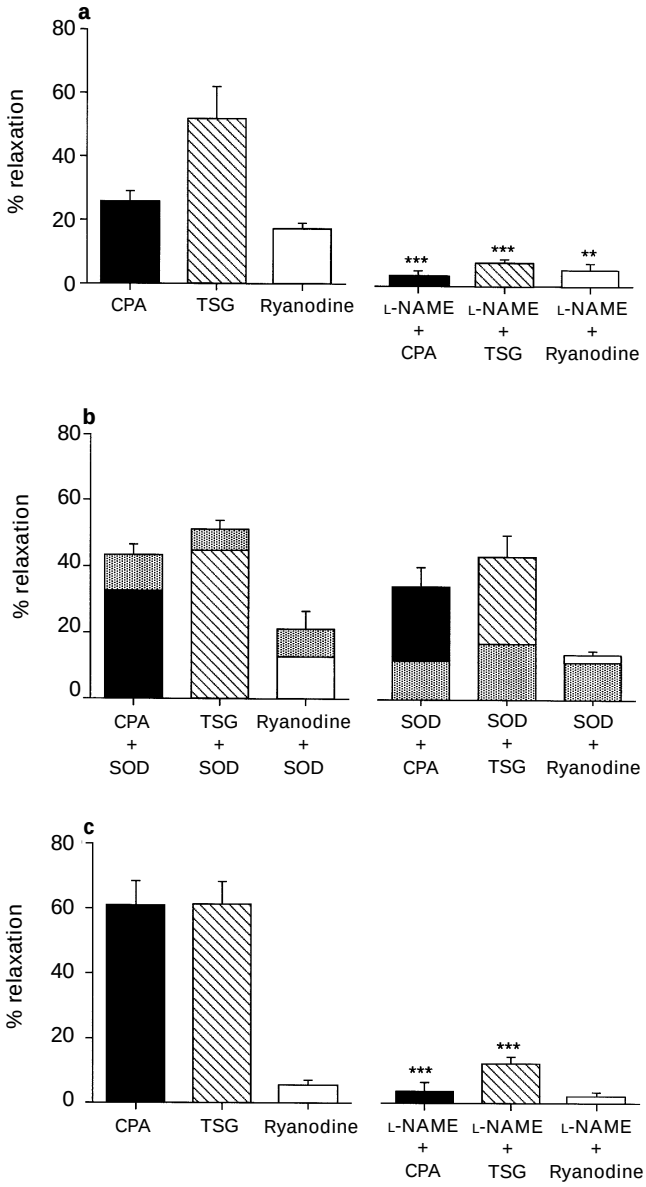

Figure 2 Direct effects of cyclopiazonic acid (CPA; solid columns, 10 μM), thapsigargin (TSG; hatched columns, 1 μM) and ryanodine (open columns, 30 μM) in cascade bioassay and isolated ring preparations. (a) All three agents induced L-NAME-sensitive relaxations of the recipient ring in cascade bioassay following addition to the donor perfusate. Relaxations to TSG were greater than those to either CPA or ryanodine. (b) Superoxide dismutase (SOD, 200 u ml^{-1}) evoked relaxations of the recipient ring (stippled areas of the columns). Relaxation to SOD was additive with that evoked by CPA regardless of the order of administration, whereas prior administration of SOD attenuated relaxations to ryanodine and TSG, and prior administration of ryanodine or TSG blunted relaxations to SOD. However, note that the magnitude of the combined relaxations to SOD/TSG or SOD/ryanodine were independent of order of administration and not significantly different from relaxations to TSG or ryanodine alone. s.e.mean values were calculated from the means of the combined relaxations to SOD and each of the three agents. (c) CPA and TSG but not ryanodine relaxed isolated aortic rings to a similar degree and again these responses were attenuated by pre-incubation with L-NAME. $^{**}P<0.01$ and $^{***}P<0.001$.

$n=7$), but these were sustained when ryanodine was removed from the perfusate (Figure 1). Increasing the concentration of ryanodine to 100 μM did not enhance these relaxations ($n=4$, data not shown). Pre-incubation of the donor aorta with L-NAME for 45 min markedly attenuated the relaxations to CPA, TSG and ryanodine (Figure 2a).

Pre-incubation with superoxide dismutase (SOD, 200 u ml^{-1}) evoked relaxations of the recipient ring of $13.3\pm1.4\%$ ($n=22$, data pooled from Figure 2b). Prior administration of SOD had no effect on the relaxation evoked by CPA, but abolished relaxations to ryanodine and attenuated the subsequent response to TSG relative to control (Figure 2b). In reciprocal experiments SOD-induced relaxations were unaffected by the presence of CPA ($11\pm3.1\%$, $n=3$), whereas they were reduced in the presence of ryanodine ($8.9\pm1.1\%$, $n=4$) and significantly attenuated by TSG ($4\pm1.5\%$, $n=4$; $P<0.01$; Figure 2b). Importantly combined relaxation to SOD plus TSG or SOD plus ryanodine was not significantly different from that observed with TSG or ryanodine alone.

Phenylephrine-indued tone (2.69 ± 0.1 g, $n=22$, data pooled from all experiments) was statistically unaffected by subsequent administration of CPA, TSG or ryanodine in isolated rings of rabbit endothelium-denuded thoracic aorta, although there were small reductions to CPA (2.37 ± 0.18 g, $n=9$) and TSG (2.32 ± 0.37 g, $n=6$) and a slight increase with ryanodine (3.01 ± 0.2 g, $n=7$). CPA and ryanodine caused a small but significant attenuation in the potency of sodium nitroprusside to relax isolated thoracic aortic rings ($n=6$ and 7; $P<0.05$, Table 1) and ryanodine caused a modest reduction in the maximal relaxation ($n=7$; $P<0.05$, Table 1). TSG exerted no

effect on the endothelium-independent relaxations to sodium nitroprusside ($n=6$, Table 1).

Effects on altered perfusate viscosity

The influence of shear stress on NO release was investigated by addition of dextran 1–4% (w/v) to the donor perfusate to increase its viscosity successively from 0.73 mPa s to 0.98, 1.24, 1.57, and 1.97 mPa s, respectively. Each increment in the concentration of dextran caused reversible relaxation of the recipient tissue which was attenuated by a 45 min pre-incubation with L-NAME (Figure 3), although a small (<10%) L-NAME-insensitive relaxation could still be observed, as previously found (Hutcheson & Griffith, 1996). Dextran (1–4%) also evoked small relaxations of isolated endothelium-intact (7.5±0.7% to 4% dextran) and endothelium-denuded (7.3±1.1% to 4% dextran) rings of thoracic aorta (NS, $n=4$ in each case). This minor non-specific effect of dextran is therefore an endothelium-independent phenomenon. Mean perfusion pressure (4.6±0.2 mmHg) was unaffected by changes in viscosity or addition of L-NAME.

Pre-incubation of the bioassay system with CPA (10 μM) or TSG (1 μM) significantly ($P<0.01$; $n=5$ for each agent) attenuated the viscosity-induced relaxations observed following addition of dextran to the perfusate (Figures 4a and 5a, respectively). In the TSG studies there was no significant difference in recipient tone before or following incubation, as the relaxation to TSG was transient (before: 2.37±0.37 g and after: 2.33±0.31 g, respectively, $n=5$). Despite the transient nature of the CPA-induced relaxation, as observed in the studies with isolated endothelium-denuded rings discussed above, there was a small difference in recipient tone

Table 1 Effect of CPA, TSG and ryanodine on sodium nitroprusside-induced relaxations of isolated rings of rabbit thoracic aorta

	EC_{50} (−log M)	*Maximal response* (%)	
Control	6.78±0.2	99.1±6.1	($n=6$)
CPA (10 μM)	6.23±0.07*	95.6±4.1	($n=6$)
Control	6.75±0.2	109.2±8.4	($n=6$)
TSG (1 μM)	6.72±0.18	97.5±4.9	($n=6$)
Control	6.76±0.14	101.9±4.5	($n=7$)
Ryanodine (30 μM)	6.26±0.17*	81.8±4.6*	($n=7$)

There was no effect of thapsigargin (TSG) on sodium nitroprusside (SNP)-induced relaxations. Both cyclopiazonic acid (CPA) and ryanodine significantly reduced the potency of sodium nitroprusside ($P<0.05$), but only ryanodine significantly reduced the maximal relaxations ($P<0.05$).

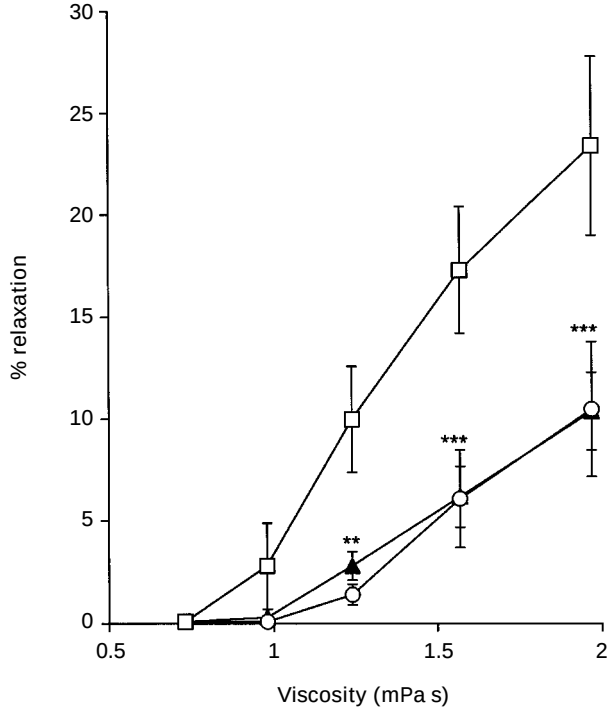

Figure 3 % relaxation of endothelium-denuded recipient rings in response to increases in the viscosity of the perfusate passing through endothelium-intact donor aortae (squares, $n=5$). Responses were significantly attenuated by L-NAME (100 μM; circles, $n=4$) implying enhanced release of NO. Following substitution of the donor vessel with rubber tubing relaxations were equivalent to those observed in the presence of L-NAME (solid triangles, $n=4$). $^{**}P<0.01$ and $^{***}P<0.001$ control vs L-NAME. Vertical lines show s.e.mean.

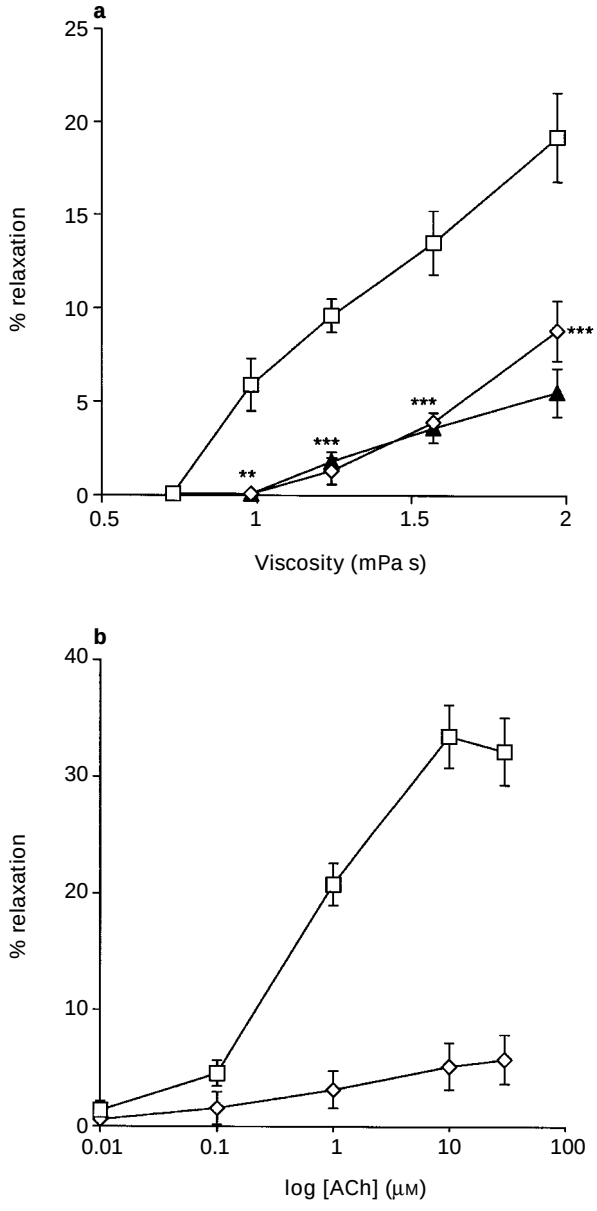

Figure 4 Cyclopiazonic acid (CPA, 10 μM, diamonds) abolished the relaxations of the recipient ring to increases in (a) perfusate viscosity ($n=7$) and (b) to acetylcholine (ACh) ($n=7$) in cascade bioassay (squares). This action can be attributed exclusively to reduced NO release as there was no effect of CPA on the residual relaxation observed with the donor vessel substituted by rubber tubing (solid triangles, $n=4$). $^{**}P<0.01$ and $^{***}P<0.001$. Vertical lines show s.e.mean.

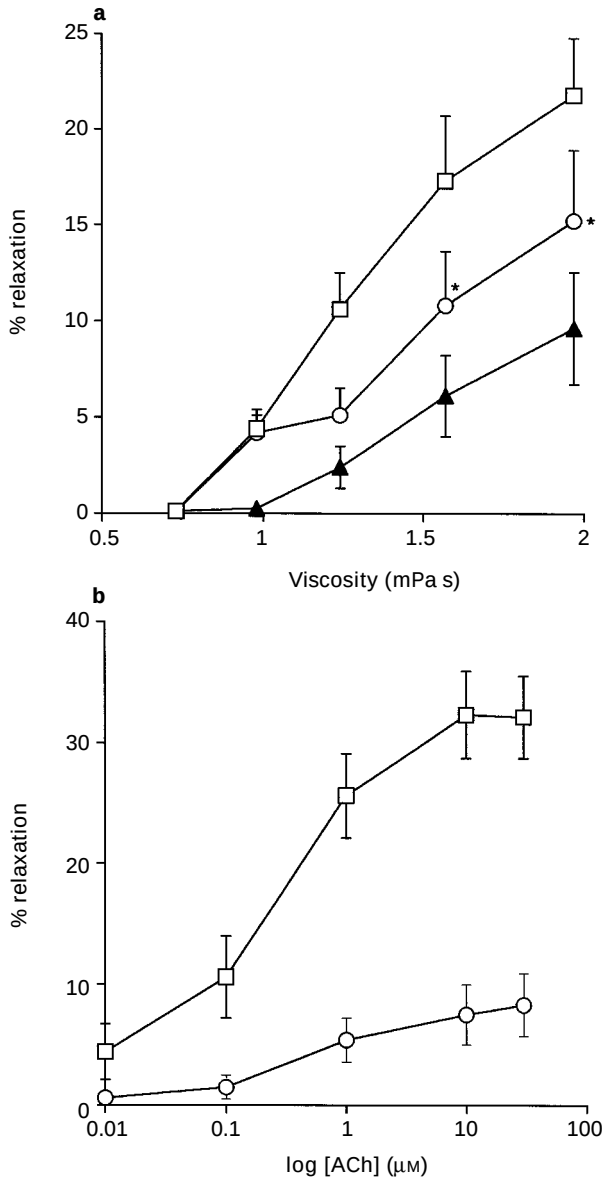

Figure 5 Thapsigargin (TSG; 1 μM, circles) significantly attenuated (a) viscosity-induced NO release (squares, $n=7$) and almost abolished (b) acetylcholine-induced relaxations (squares, $n=7$). There was again no effect of this agent on the direct relaxation to dextran (solid triangles, $n=4$). *$P<0.05$. Vertical lines show s.e.mean.

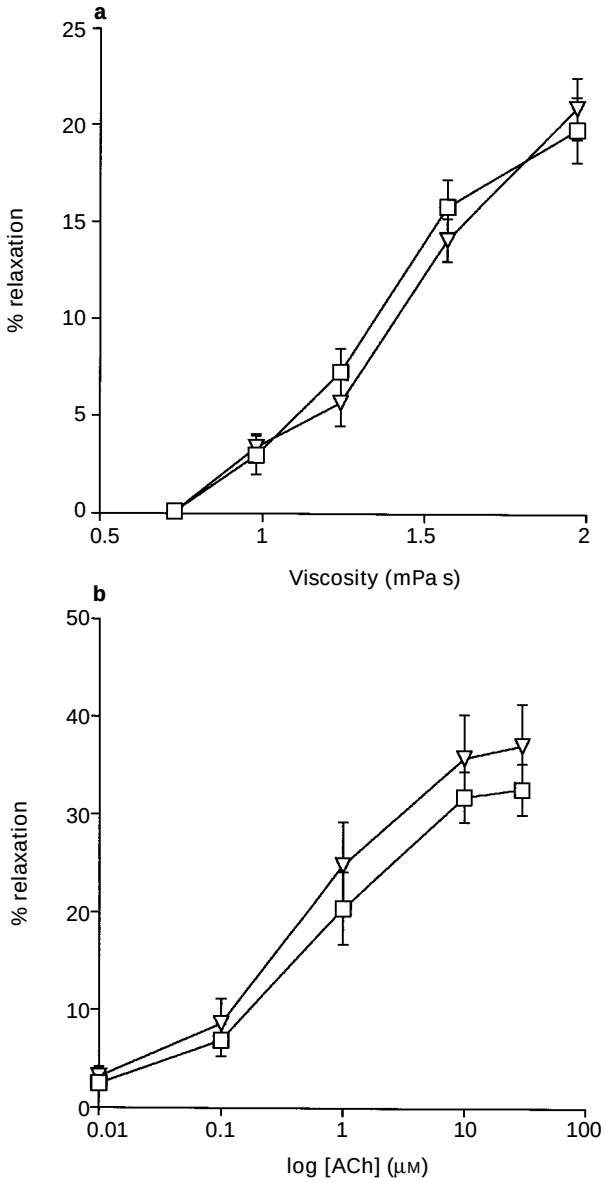

Figure 6 Incubation with ryanodine (30 μM, triangles) had no effect on (a) viscosity- or (b) acetylcholine-induced NO release (squares, $n=6$ in both cases). Vertical lines show s.e.mean.

before and after CPA incubation, the values being 2.36 ± 0.21 g and 2.16 ± 0.25 g, respectively ($n=6$, $P<0.05$). This may be attributable to the direct effect of DMSO on the recipient ring which was present throughout the CPA studies but not in experiments with TSG. CPA and TSG did not influence the magnitude of the non-specific relaxation of the recipient ring induced by dextran (eg) when the donor vessel was replaced with rubber tubing (Figures 4a and 5a). Ryanodine (30 μM) had no effect on the viscosity-related responses (Figure 6a) and increasing the concentration of ryanodine to 100 μM also had no effect (data not shown). The recipient tone was slightly lower after incubation with 30 μM ryanodine (before: 3.35 ± 0.25 g, after: 3.08 ± 0.35 g, $n=6$, $P<0.05$) due to its ability to amplify the effects of basal NO activity.

Effects on acetylcholine-induced responses

To investigate agonist-evoked NO release cumulative concentration-response curves to acetylcholine (10 nM – 30 μM) were constructed. Addition of acetylcholine to the donor perfusate induced relaxations of the recipient ring that could be completely abolished by 45 min pre-incubation of the donor with L-NAME (100 μM). Incubation of vessels with cyclopiazonic acid (10 μM) and thapsigargin (1 μM) markedly attenuated acetylcholine-induced NO release ($n=6$ and $n=5$, Figures 4b and 5b, respectively). Recipient tone before and after treatment with TSG was unchanged being 2.37 ± 0.38 g and 2.37 ± 0.33 g ($n=5$), whereas after incubation with CPA recipient tone showed a small but significant reduction from 2.31 ± 0.22 g to 2.01 ± 0.27 g ($n=6$, $P<0.05$), again as a possible consequence of the direct effect of DMSO on the recipient ring. Ryanodine (30 μM) had no effect on acetylcholine-induced responses (Figure 6b) either in terms of the EC_{50} (control: 2.4 ± 0.64 μM; ryanodine: 1.2 ± 0.3 μM, $n=6$) or the maximal response (control: $30.8\pm8.4\%$; ryanodine: $35.8\pm8.4\%$, $n=6$). A similar lack of effect was observed when the concentration of ryanodine was increased to 100 μM (data not shown). Again there was a small but significant difference in recipient tone before and after incubation with 30 μM ryanodine (3.35 ± 0.25 and 3.17 ± 0.34, respectively, $n=6$; $P<0.05$).

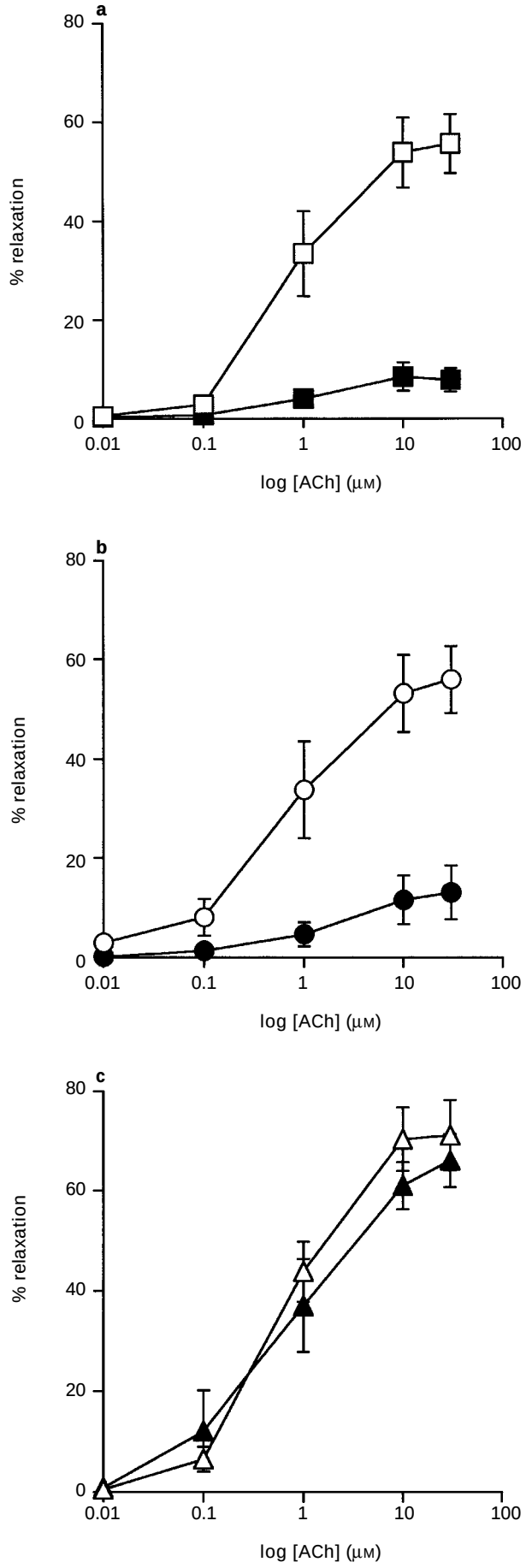

Figure 7 Effects of (a) cyclopiazonic acid (CPA; 10 μM, solid squares), (b) thapsigargin (TSG; 1 μM, solid circles) and (c) ryanodine (30 μM, solid triangles) on acetylcholine (ACh)-induced relaxations of isolated rings of rabbit abdominal aorta. CPA ($n=6$) and TSG ($n=6$) but not ryanodine ($n=7$) markedly attenuated the response to acetylcholine. Control, open symbols. Vertical lines show s.e.mean.

Inclusion of 1% or 2% dextran in the perfusate had no effect on acetylcholine-induced relaxations either in terms of the EC_{50} (control: 0.85 ± 0.4 μM, $n=7$; 1% dextran: 0.94 ± 0.42 μM, $n=6$; 2% dextran: 0.67 ± 0.07 μM, $n=3$) or the maximal response (control: 29.6 ± 4.8%, $n=7$; 1% dextran: 28.6 ± 3.8%, $n=6$; 2% dextran: 29.9 ± 4.4%, $n=3$).

Isolated arterial ring studies

Cumulative concentration-response curves to acetylcholine (10 nM – 30 μM) and sodium nitroprusside (1 nM – 30 μM) were constructed to investigate endothelium-dependent and -independent responses, respectively. Acetylcholine induced L-NAME-sensitive relaxations of abdominal aortic rings, confirming their dependence on NO synthesis (not shown). CPA and TSG induced L-NAME-sensitive transient relaxations of the isolated rings (61 ± 3.8%, $n=9$ and 61.3 ± 7%, $n=5$ respectively, Figure 2c) which returned to baseline within approximately 1 h, whereas ryanodine exerted no effect (not illustrated). In marked contrast to the cascade bioassay studies also, co-incubation with SOD (200 u ml^{-1}) had no effect on either CPA- or TSG-induced relaxations (59 ± 9.1%, $n=4$ and 66.2 ± 11.5%, $n=4$, respectively) in experiments in which these agents were allowed to incubate for approximately 1 h to allow ring tone to return towards pre-drug levels. Consequently, there was no significant difference in the ring tone before and following incubation with TSG (before 2.36 ± 0.12 g, after 2.32 ± 0.37 g, $n=6$) and ryanodine (before 2.82 ± 0.15 g, after 3.01 ± 0.2 g, $n=7$). There was a small difference following incubation with CPA (before 2.81 ± 0.18 g, after 2.34 ± 0.18 g, $n=9$) but this was significant only at the $P<0.1$ level. As in cascade bioassay studies, CPA and TSG almost abolished acetylcholine-induced NO release ($n=7$ for each study; Figure 7) but ryanodine exerted no significant effect on the EC_{50} (control: 0.88 ± 0.24 μM; ryanodine: 1.7 ± 0.7 μM, $n=7$) or maximal response (control: 68.3 ± 4.8%; ryanodine: 64.5 ± 4.8%, $n=7$; Figure 7).

Discussion

We have investigated the contribution of Ca^{2+} release from endothelial stores to agonist- and flow-induced NO release from rabbit abdominal aortae in cascade bioassay and agonist-induced relaxation of isolated rings. The possible contribution of prostanoids was excluded by the presence of indomethacin in all experiments. Cyclopiazonic acid (CPA) and thapsigargin (TSG), inhibitors of the endoplasmic reticulum Ca^{2+}-ATPase, were employed to deplete the intracellular stores, and ryanodine was used to investigate the involvement of the ryanodine-sensitive Ca^{2+} release channel in the signalling process. The findings of this present study are consistent with the conclusions that: (1) the release of Ca^{2+} from internal stores is involved in both agonist- and flow-induced NO release, but that (2) Ca^{2+}-induced Ca^{2+} release (CICR) through the ryanodine receptor/Ca^{2+} release channel is not involved in the signalling process associated with NO production in response to either stimulus.

Role of intracellular Ca^{2+} stores in acute flow- and acetylcholine-induced NO release

TSG and CPA both induced relaxations in cascade bioassay and endothelium-intact isolated rings in organ bath experiments which were attributable solely to direct release of NO as they were abolished by L-NAME. Their effects were not secondary to enhanced sensitivity of vascular smooth muscle to NO, as TSG did not alter the EC_{50} value for sodium

nitroprusside (SNP) and CPA actually caused a small decrease in potency. This is in agreement with previous findings showing that acute treatment with TSG has no effect on either nitroglycerin (GTN)- or SNP-induced relaxation of rabbit aorta (Macarthur *et al*., 1993; Amerini *et al*., 1996), whereas CPA attenuates relaxations to GTN in the same vessel type (Luo *et al*., 1993). However, it should be noted that prolonged (5 h) incubation with TSG has been shown to reduce GTN-induced relaxations in rabbit aorta by up to 30% (Luo *et al*., 1993).

Observations that superoxide dismutase (SOD) was without effect on either CPA- or TSG-induced relaxations in isolated rings reflect the short diffusion time for NO between the endothelium and the smooth muscle in intact preparations, and contrasts with the greater relaxation found in cascade bioassay. The magnitude of the relaxation to CPA, observed in cascade bioassay, was unaffected by prior incubation with SOD whereas relaxation to TSG was attenuated. Conversely, administration of SOD after CPA still resulted in relaxation whereas this small additional response was significantly attenuated in the presence of TSG. These observations suggest that the response to CPA is solely mediated by direct stimulation of NO production, whereas TSG also attenuates destruction of NO by the superoxide anion. Our observations with the cascade bioassay do not permit distinction between direct scavenging of the superoxide radical by TSG, intracellular inhibition of superoxide production, or both, although TSG is known to inhibit superoxide production in response to Ca^{2+} mobilization by arachidonic acid in human neutrophils (Hardy *et al*., 1995). The ability of TSG to act as a modulator of free radical interactions explains why relaxations to TSG were greater than those to CPA in cascade bioassay, whereas they were equivalent in ring preparations. Indeed, following prior administration of SOD, relaxations to TSG and CPA in cascade bioassay were statistically similar, thus suggesting equivalent direct effects of NO production at the concentrations employed.

Our findings confirm previous data that NO production by endothelial cells is stimulated by depletion of internal stores with CPA or TSG (Moritoki *et al*., 1994; Zheng *et al*., 1994; Buckley *et al*., 1995), probably as a consequence of the activation of the capacitative Ca^{2+} influx pathway (Schilling *et al*., 1992; Dolor *et al*., 1993; Yamamoto *et al*., 1995; Pasyk *et al*., 1995). They also confirm findings that NO release in response to TSG and CPA may be transient, declining towards basal levels within an hour (Macarthur *et al*., 1993; Zheng *et al*., 1994). Furthermore, as the endothelial NO synthase is a Ca^{2+}-dependent enzyme, our findings are consistent with findings that the $[Ca^{2+}]_i$ response to 10 μM CPA in human aortic endothelial cells is transient, declining rapidly to baseline within 15 min (Hosoki & Iijima, 1995). The endothelial $[Ca^{2+}]_i$ response to 1 μM TSG is also transient reaching a peak after 2–3 min and declining slowly to approximately 35–45% after 15–30 min (Macarthur *et al*., 1993; Yamamoto *et al*., 1995). These findings suggest that endothelial $[Ca^{2+}]_i$ levels before and after addition of both TSG and CPA in our studies will be comparable since there was a time lapse of at least 1 h before repetition of viscosity and acetylcholine responses. The transient nature of these responses also means that recipient tone following drug addition does not differ greatly from pre-drug values, although small differences were apparent in the CPA studies, probably due to a direct effect of the vehicle DMSO on the recipient ring.

TSG has previously been shown to abolish agonist-evoked NO production in bovine cultured aortic endothelial cells and intact rabbit thoracic aorta and femoral artery (Macarthur *et al*., 1993; Amerini *et al*., 1996). This is consistent with our findings with CPA and TSG against acetylcholine-induced NO release in cascade bioassay and isolated rings. However, Macarthur *et al*. (1993) concluded that TSG was without effect on shear-related NO production on the basis of studies in which the applied shear stress was estimated as being of the order of 0.12–0.55 dyn cm^2. Our studies employed viscosities slightly below that of blood *in vivo* (2.5–6 mPa s, depending on shear rate) at flow rates which generate intimal shear stresses of the order of $\sim$10 dyn cm^2 (calculated on the basis of diameter 1.5–2 mm, flow rate 9 ml min^{-1}, and viscosity 2 mPa s) (Hutcheson & Griffith, 1996). At these higher shear stresses, which are within the range normally found in small conduit arteries, TSG and CPA attenuated NO release, suggesting that Ca^{2+} release from intracellular stores plays an important role in flow-induced NO production under physiological conditions where there may be acute changes in flow. Given the 20–100 fold differences in applied shear stress, the apparent lack of effect of TSG on flow-induced NO release in the studies of Macarthur *et al*. (1993) may be attributable to the ability of TSG to enhance basal NO activity through its putative effects on superoxide radicals.

We excluded the possibility that shear-related NO production detected in cascade bioassay on increasing perfusate viscosity was influenced by altered convective diffusion in the intimal boundary layer adjacent to the endothelium, i.e. by secondary changes in the net rate of NO destruction by superoxide anions present in the perfusate or generated by the endothelium. Concentration-relaxation curves to acetylcholine were thus unaffected by addition of dextran to the perfusate, suggesting that convective effects are not significant under the experimental conditions employed. Furthermore, the experiments of Tesfamarian and Cohen (1988) confirm that dextran enhances abluminal NO release towards the media of the vessel wall through shear related mechanisms. Dilatation of isolated perfused arterial segments evoked by dextran was abolished either by co-administration of methylene blue (an inhibitor of soluble guanylate cyclase) or endothelial removal. However, it should also be noted that in the present study dextran induced both a L-NAME-sensitive and a smaller L-NAME-insensitive relaxation in cascade bioassay. Control experiments showed that the L-NAME-insensitive component was unaffected when the donor vessel was substituted by rubber tubing and was therefore mediated by a direct effect on the recipient smooth muscle. This conclusion was further supported by the endothelium-independent nature of dextran-induced relaxation of isolated thoracic aortic rings. The mechanism underlying this secondary non-specific action of dextran remains to be elucidated.

Role of the ryanodine receptor in acute flow- and acetylcholine-induced NO release

There is a substantial body of evidence that indicates the presence of more than one type of intracellular Ca^{2+} store in endothelial cells. Freay *et al*. (1989) demonstrated that IP_3 released only 75% of the non-mitochondrial fraction of the Ca^{2+} store in bovine cultured aortic endothelial cells, suggesting the existence of a second IP_3-insensitive store. In cultured endothelial cells derived from a variety of vessel types it has been demonstrated that caffeine, which sensitizes the endoplasmic reticulum Ca^{2+} release channel and enhances Ca^{2+}-induced Ca^{2+} release (CICR), can mobilize Ca^{2+} from an internal store distinct from that sensitive to IP_3 although some functional overlap between the two may exist (Buchan & Martin, 1991; Thuringer & Sauve, 1992; Adams *et al*., 1993; Rusko *et al*., 1993; 1995; Corda *et al*., 1995). Ryanodine also binds to the caffeine-sensitive Ca^{2+} channel and may thereby inhibit caffeine-induced $[Ca^{2+}]_i$ transients in rabbit aortic endothelium (Rusko *et al*., 1995). It is also itself capable of inducing Ca^{2+} mobilization and elevating cytosolic $[Ca^{2+}]$ in endothelial cells by locking the channel in an open sub-conductance state (Ziegelstein *et al*., 1994). Anti-ryanodine receptor antibody binding sites have been found surrounding the nucleus and distributed in a nonhomogeneous fashion throughout the cytosol of vascular and endocardial endothelium (Lesh *et al*., 1993).

In the present study we found that ryanodine exerted no effect in isolated rings while nevertheless evoking L-NAME-sensitive relaxations of the recipient ring in cascade bioassay.

These relaxations were sustained following washout of ryanodine from the donor perfusate for up to 1 h, and are therefore likely to reflect the irreversible nature of its binding to the Ca^{2+} release channel (Ziegelstein *et al*., 1994; Rusko *et al*., 1995). This response cannot be attributed to a direct action of ryanodine on the recipient smooth muscle as its effects on SNP-induced relaxations were minimal. Indeed, ryanodine-induced relaxations in cascade bioassay were abolished by prior administration of SOD and, thus, appear to result from an irreversible reduction in free radical activity which prolongs the action of NO rather than enhances its production through elevated endothelial $[Ca^{2+}]_i$. Despite this apparent ability to reduce free radical activity and its ability to deplete acetylcholine-sensitive Ca^{2+} stores in rabbit freshly isolated aortic endothelial cells (Wang *et al*., 1995), ryanodine had no effect on either viscosity-related or acetylcholine-induced NO release. Ca^{2+}-induced Ca^{2+} release via the ryanodine receptor thus plays no significant role in NO release in response to either activation of specific receptors or mechanical stimulation in rabbit aorta.

In conclusion, we have confirmed that CPA and TSG can induce release of NO from endothelial cells and have further shown that release of Ca^{2+} from intracellular stores plays an important role in both acetylcholine- and acute shear stress-evoked NO release in rabbit abdominal aorta. We have also demonstrated in both cascade bioassay and isolated rings that Ca^{2+}-induced Ca^{2+} release through the ryanodine-sensitive Ca^{2+} release channel plays no direct role in acetylcholine- or flow-induced NO release.

This work was funded by the Medical Research Council.

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(Received December 19, 1996
Revised May 6, 1997
Accepted June 2, 1997)