British Journal of Pharmacology (2002) 137, 1071–1081

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Inhibition of rat platelet aggregation by the diazeniumdiolate nitric oxide donor MAHMA NONOate

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1 Inhibition of rat platelet aggregation by the nitric oxide (NO) donor MAHMA NONOate (*Z*-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazen-1-ium-1,2-diolate) was investigated. The aims were to compare its anti-aggregatory effect with vasorelaxation, to determine the effects of the soluble guanylate cyclase inhibitor, ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), and to investigate the possible role of activation of sarco-endoplasmic reticulum calcium-ATPase (SERCA), independent of soluble guanylate cyclase, using thapsigargin.

2 MAHMA NONOate concentration-dependently inhibited sub-maximal aggregation responses to collagen (2–10 μg ml^{-1}) and adenosine diphosphate (ADP; 2 μM) in platelet rich plasma. It was (i) more effective at inhibiting aggregation induced by collagen than by ADP, and (ii) less potent at inhibiting platelet aggregation than relaxing rat pulmonary artery.

3 ODQ (10 μM) caused only a small shift (approximately half a log unit) in the concentration-response curve to MAHMA NONOate irrespective of the aggregating agent.

4 The NO-independent activator of soluble guanylate cyclase, YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole; 1–100 μM), did not inhibit aggregation. The cGMP analogue, 8-pCPT-cGMP (8-(4-chlorophenylthio)guanosine 3′5′ cyclic monophosphate; 0.1–1 mM), caused minimal inhibition.

5 On collagen-aggregated platelets responses to MAHMA NONOate (ODQ 10 μM present) were abolished by thapsigargin (200 nM). On ADP-aggregated platelets thapsigargin caused partial inhibition.

6 Results with S-nitrosoglutathione (GSNO) resembled those with MAHMA NONOate. Glyceryl trinitrate and sodium nitroprusside were poor inhibitors of aggregation.

7 Thus inhibition of rat platelet aggregation by MAHMA NONOate (like GSNO) is largely ODQ-resistant and, by implication, independent of soluble guanylate cyclase. A likely mechanism of inhibition is activation of SERCA.

British Journal of Pharmacology (2002) **137,** 1071–1081. doi:10.1038/sj.bjp.0704971

Keywords: MAHMA NONOate; nitric oxide donor drugs; ODQ; ODQ-resistant responses; rat platelet aggregation; sarco-endoplasmic reticulum calcium-ATPase; soluble guanylate cyclase inhibitor

Abbreviations: ANOVA, analysis of variance; DMSO, dimethylsulphoxide; GSNO, S-nitrosoglutathione; MAHMA NONOate, *Z*-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazen-1-ium-1,2-diolate; NONOates, diazeniumdiolates; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine 3′5′ cyclic monophosphate; PPP, platelet poor plasma; PRP, platelet rich plasma; PSS, physiological salt solution; SERCA, sarco-endoplasmic reticulum calcium-ATPase; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole

Introduction

NONOates, or 1-substituted diazen-1-ium-1,2-diolates, are a novel group of nitric oxide (NO) donor drugs that are complexes of NO bound to a nucleophile (Maragos *et al*., 1991). These compounds are characterized by their ability to generate NO 'spontaneously' at physiological pH, without requiring tissue activation (Feelisch & Stamler, 1996). They have proved to be reliable sources of NO both *in vitro* and *in vivo* (Fitzhugh & Keefer, 2000). Hence these drugs may have potential therapeutic value in the treatment of cardiovascular diseases where NO gas and/or other NO donor drugs are currently used e.g. pulmonary hypertension (Neonatal inhaled nitric oxide study group, 1997; Davidson *et al*., 1998), angina pectoris (Schonafinger, 1999; Marsh & Marsh, 2000), heart failure (Abrams, 1996) and hypertension (Abrams, 1996; Vaughan & Delanty, 2000). In these clinical conditions NO donor drugs are used primarily for their vasodilator properties, i.e. their ability to relax vascular smooth muscle cells. However, an ability to inhibit platelet activation (aggregation, adhesion and/or degranulation) may be an additional therapeutic benefit of some NO donor drugs.

The comparative effects of NONOates on blood vessels *vs* platelets have not been systematically studied, and the mechanism(s) of action on platelets remains to be elucidated. The relative abilities of NO donor drugs to cause vasorelaxation compared with their abilities to inhibit platelet aggregation vary between different NO donors (Sogo *et al*., 2000a, b), as do their mechanisms of action, especially with respect to the contribution of cGMP (Homer *et al*., 1999; Feelisch *et al*., 1999; Garcia-Pascual *et al*., 1999; Homer & Wanstall, 2000; Tseng *et al*., 2000). Most of the work on the mechanisms of action of NO donors has been conducted in

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blood vessels, but data obtained in other tissues indicate that the mechanisms vary between tissue types (Ellis, 1997; Abi-Gerges *et al*., 2001; Alcon *et al*., 2001; Buxton *et al*., 2001). Previous biochemical studies have shown that, in platelets, NO donor drugs can increase cGMP (Becker *et al*., 2000). Also, in platelets, NO donors have been shown to inhibit elevations in free intracellular calcium (Geiger *et al*., 1992; Ivanova *et al*., 1993; Sage *et al*., 2000), an important component of agonist-induced platelet activation (Jin *et al*., 1998; Shiraishi *et al*., 2000). One study on calcium measurements in human platelets has provided evidence for direct activation of sarco-endoplasmic reticulum calcium-ATPase (SERCA) by NO gas, independently of cGMP (Trepakova *et al*., 1999). However it is not known whether these mechanisms apply to NONOates and, importantly, whether the data in these biochemical experiments translate into a functional effect.

In this study the effects of MAHMA NONOate have been examined on one of the functional responses in platelets, *viz*. aggregation. The aims of the study were to (i) compare its anti-aggregatory effect with vasorelaxation, (ii) determine the role of soluble guanylate cyclase using the selective inhibitor, ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; Garthwaite *et al*., 1995), and (iii) determine the role, if any, of SERCA activation, independent of soluble guanylate cyclase, using the SERCA inhibitor thapsigargin. We have compared MAHMA NONOate with representative drugs from three other classes of NO donor, *viz*., S-nitrosoglutathione (GSNO; an S-nitrosothiol), sodium nitroprusside and glyceryl trinitrate. Rat platelets were used so that the results could be compared with our previous data on rat pulmonary artery (Homer & Wanstall, 2000) without introducing a variation in species, and also because the effects of ODQ have not previously been examined on rat platelets. Two different physiologically relevant aggregating agents were used, *viz*. collagen and ADP.

A preliminary account of these data was presented to a meeting of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists, Dunedin, New Zealand, December 2001 (Homer & Wanstall, 2001).

Methods

Male Wistar rats (body weight 342 ± 5 g, $n = 127$) were anaesthetised with pentobarbitone (90 mg kg^{-1}, i.p.) and the abdomen and/or the thorax was opened. Seven ml of blood were obtained from the abdominal aorta, *via* a heparinized cannula, and/or the main pulmonary artery was removed.

Measurement of inhibition of platelet aggregation

Platelet preparations Blood was collected into a syringe containing 105 IU of heparin sodium, giving a final concentration of 15 IU ml^{-1} blood. The blood was centrifuged at $200 \times g$ for 10 min and the top layer, platelet rich plasma (PRP), was removed. In some experiments the PRP from two rats was pooled. Platelet poor plasma (PPP) was prepared by centrifuging the remaining sample at $4000 \times g$ for 15 min. In all experiments the PPP from two rats was pooled. The number of platelets in the PRP sample was determined microscopically using a haemocytometer (mean platelet count $1.84 \pm 0.04 \times 10^6$ platelets μl^{-1}; $n = 80$). The PRP was then diluted first with PPP to give 6×10^5 platelets μl^{-1} and then 1 : 1 with normal saline.

Platelet aggregation was determined in diluted PRP (see above) using a turbidimetric aggregometer (Chrono-log Corporation, PA, U.S.A.). Contents of the cuvettes (a total volume of 500 μl, including drugs) in the aggregometer were maintained at 37°C and stirred constantly at 1200 r.p.m. Changes in light transmission through the diluted PRP were recorded on a chart recorder (Rikadenki; Tokyo, Japan). Light transmission through diluted PRP and PPP (diluted 1 : 1 with normal saline) were used for calibration, and represented minimum (0%) and maximum (100%) light transmission, respectively.

Experimental protocol and data analysis In preliminary experiments concentration-response curves to the aggregating agents collagen and ADP were obtained. Aggregation responses were defined as the difference in light transmission through diluted PRP after and before the addition of collagen or ADP and were expressed as a percentage of maximal light transmission, i.e. as a percentage of: (light transmission (PPP) − light transmission (PRP)). Maximal aggregation responses were: collagen $107 \pm 2.3\%$; ADP $91 \pm 3.4\%$; $n = 4$. Concentrations that induced sub-maximal platelet aggregation were determined i.e. collagen (2–10 μg ml^{-1}; $84 \pm 1.1\%$ aggregation; $n = 42$) or ADP (2 μM; $79 \pm 0.8\%$ aggregation; $n = 40$), and used in the following experiments unless stated otherwise.

The inhibitory effects of several NO donor drugs or the NO-independent activator of soluble guanylate cyclase, YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole), or the membrane permeable cGMP analogue, 8-pCPT-cGMP (8-(4-chlorophenylthio)guanosine 3′5′ cyclic monophosphate), or the nucleophile MAHMA, were examined on sub-maximal aggregation responses. The inhibitory drugs were added to the cuvette and incubated for the following times before the addition of aggregating agent: MAHMA NONOate, MAHMA or GSNO, 2 min; sodium nitroprusside or glyceryl trinitrate, 5 min; YC-1, 3 min; 8-pCPT-cGMP, 10 min. Control responses were obtained in the presence of drug vehicle only. The inhibitory responses were calculated as:

$$\frac{\text{aggregation response (control)} - \text{aggregation response (drug present)}}{\text{aggregation response (control)}} \times 100\%$$

Potency was defined as the negative logarithm of the IC_{50} (where IC_{50} is the concentration producing 50% inhibition of platelet aggregation) and potency values were interpolated from individual concentration-response curves. IC_{50} values were obtained only when collagen was the aggregating agent because in the ADP experiments 50% inhibition was not always achieved.

In some experiments the effects of the following antagonist drugs were examined as described: (a) Concentration-response curves to MAHMA NONOate and GSNO were obtained in the absence and presence of the soluble guanylate cyclase inhibitor, ODQ (incubation time 30 min). 10 μM ODQ was used in most of these experiments because this, or a lower, concentration has been shown to (i) maximally inhibit purified soluble guanylate cyclase stimulated by NO donor drugs (Garthwaite *et al*., 1995; Schrammel *et al*.,

1996), (ii) completely prevent NO donor drug-induced elevations in cGMP in platelets (Moro *et al*., 1996) and (iii) maximally inhibit the effects of NO donors in blood vessels (Homer *et al*., 1999; Homer & Wanstall, 2000). Additional experiments were carried out with 1, 3, 10 and 30 μM ODQ. In these latter experiments the effects of these concentrations of ODQ were expressed as 'per cent inhibition of the control response to MAHMA NONOate', calculated as:

$$\frac{\text{MAHMA NONOate response (no ODQ)} - \text{MAHMA NONOate response (ODQ)}}{\text{MAHMA NONOate response (no ODQ)}} \times 100\%$$

(b) Concentration-response curves to MAHMA NONOate, on collagen-aggregated platelets, were obtained in the absence and presence (incubation time 1 min) of superoxide dismutase (150 IU ml^{-1}) plus catalase (1200 IU ml^{-1}) to remove superoxide. (c) Responses to selected concentrations of MAHMA NONOate (1 or 3 μM) or GSNO (10 or 100 μM) were obtained in the absence and presence of the SERCA inhibitor thapsigargin (200 nM; incubation time 5 min). In this series of experiments ODQ (10 μM) was present throughout. Thapsigargin did not cause aggregation but it did slightly increase the aggregation responses to collagen and ADP. Therefore additional control experiments, without thapsigargin, were carried out in which these increases in the aggregation responses were mimicked by raising the concentration of aggregating agent (see Results).

Measurement of vasorelaxation

Artery preparations Main pulmonary artery was cleared of adhering connective tissue and from it a single ring preparation, 3 mm in length, was obtained. In some experiments the endothelium was removed by gently rubbing the lumen of the preparation with forceps. The preparations were mounted around two stainless steel wires in a vertical organ bath containing physiological salt solution (PSS) at 37°C and gassed with 95% O_2/5% CO_2. The composition of the PSS was (mM): NaCl 118, KCl 5.9, $CaCl_2$ 1.5, $MgSO_4$ 0.72, $NaHCO_3$ 25, glucose 11.7, Na_2EDTA 0.025. The preparations were stretched to a resting force of 10 mN. Changes in force in the circular muscle were recorded isometrically with a Statham Universal Transducer (UC3+UL5) attached to a micrometer (Mitutoyo, Tokyo, Japan).

Experimental protocol and data analysis All preparations were allowed to equilibrate for 1 h (PSS replaced at 15 min intervals). The absence or presence of the endothelium was confirmed by contracting the preparations sub-maximally with phenylephrine (0.1 μM) and, once the contraction reached a plateau, adding acetylcholine (1 μM). In those preparations in which the endothelium was not removed the relaxation response to acetylcholine was 55±3.8% reversal of the phenylephrine contraction (n=20) confirming that the endothelium was functional. In those preparations in which the endothelium was removed, acetylcholine caused no relaxation but instead a small contraction (6±0.6% of the contraction to phenylephrine; n=4), but no relaxation, confirming the absence of functional endothelium. A contraction to potassium-depolarizing PSS (in which 80 mM NaCl was replaced with 80 mM KCl) was then obtained to stabilize the preparation. The tissues were washed thoroughly, pre-contracted with phenylephrine (0.1 μM) and a relaxation concentration-response curve to MAHMA NONOate, GSNO, sodium nitroprusside, glyceryl trinitrate, YC-1 or 8-pCPT-cGMP was obtained. The experiments with the NO donor drugs were carried out on endothelium-intact preparations because, on rat pulmonary artery, responses to NO donors are not affected by endothelium removal (i.e. they are neither endothelium-dependent nor potentiated in the absence of endothelium; Wanstall *et al*., 1997; Homer & Wanstall, 1998; 2000; Homer, unpublished). YC-1, however, was studied on endothelium-denuded preparations because, in contrast to the NO donor drugs, part of the response is reported to be endothelium-dependent (Galle *et al*., 1999; Feelisch *et al*., 1999). Responses to the relaxant drugs were measured from the plateau of the phenylephrine contraction and were expressed as 'per cent reversal' of the phenylephrine contraction. Where appropriate, the IC_{50} (concentration giving 50% reversal of the phenylephrine contraction) was interpolated from the plot of response *vs* log concentration of relaxant drug.

Drugs and solutions

Sources of drugs were as follows: Collagen (equine tendon) and ADP (Helena Laboratories, U.S.A.); glyceryl trinitrate and heparin sodium (David Bull Laboratories, Australia; ampoules); MAHMA NONOate and YC-1 (Cayman, U.S.A.); thapsigargin (Biomol, U.S.A.); pentobarbitone sodium (Merial, Australia); all other drugs (Sigma-Aldrich, Australia). Solutions of drugs were prepared as follows: acetylcholine (10 mM), catalase (120,000 IU ml^{-1}), sodium nitroprusside (10 mM) and superoxide dismutase (15,000 IU ml^{-1}) in deionized water; MAHMA NONOate (10 mM) in 10 mM NaOH; GSNO (10 mM) in 0.1 mM HCl; phenylephrine (10 mM) in 10 mM HCl; ODQ (10 mM), 8-pCPT-cGMP (100 mM), thapsigargin (10 mM) and YC-1 (10 mM) in dimethylsulphoxide (DMSO). ADP (0.5 mM) in 0.9 % saline. Ampoules of glyceryl trinitrate contained 22 mM in ethanol. Collagen was supplied in solution (100 μg ml^{-1}). Dilutions, when required, were made as follows: MAHMA NONOate in 10 mM NaOH; GSNO in 0.1 mM HCl; ADP, glyceryl trinitrate (platelet experiments) and ODQ in 0.9% saline; catalase, sodium nitroprusside (platelet experiments), superoxide dismutase and thapsigargin in deionized water; 8-pCPT-cGMP and YC-1 in DMSO. In the artery preparation experiments acetylcholine, glyceryl trinitrate, phenylephrine and sodium nitroprusside were diluted in PSS. During the experiments drug dilutions were kept on ice and dilutions of NO donors were protected from light. Concentrations of glyceryl trinitrate, YC-1 or 8-pCPT-cGMP that contained concentrations of ethanol or DMSO of >0.5 and $>1\%$ v v^{-1}, respectively, were not used as at these concentrations the vehicles inhibited collagen-induced aggregation.

In order to examine the effects of the nucleophile, MAHMA, a solution of MAHMA NONOate (200 mM in 1 M HCl) was allowed to decompose (Homer & Wanstall, 1998). The solution was neutralized (pH 7) with 1 M NaOH before use.

Statistics

Mean values were calculated from data obtained from a number (n) of different preparations and are quoted with their s.e.mean. Differences between mean values have been assessed by paired or unpaired t-test. Differences between complete concentration-response curves have been analysed by two-way analysis of variance (ANOVA).

Results

Inhibition of platelet aggregation: comparison with pulmonary vasorelaxation

MAHMA NONOate caused concentration-dependent inhibition of platelet aggregation induced by either collagen or ADP (Figure 1a). It was more effective at inhibiting aggregation to collagen than to ADP, i.e. there was a significant difference in maximum responses (% inhibition; collagen 93 ± 0.8, $n=5$; ADP 47 ± 3.2, $n=6$; $P<0.001$; unpaired t-test). MAHMA NONOate was less potent as an inhibitor of aggregation than as a pulmonary vasorelaxant (Figure 1a). This difference in potency, based on values of negative log IC_{50} obtained in pulmonary artery and collagen-aggregated platelets, was between one and two orders of magnitude (see relative potency data in Table 1). The nucleophile, MAHMA, had no effect on aggregation responses at concentrations up to 1 mM ($n=3$; data not shown).

Comparison with other NO donors GSNO, sodium nitroprusside and glyceryl trinitrate likewise caused concentration-dependent inhibition of aggregation and were more effective against collagen than against ADP (Figure 1b–d). Maximum inhibitory responses for GSNO were: collagen $74 \pm 3.4\%$, $n=6$; ADP $46 \pm 4.8\%$, $n=6$ ($P<0.001$, unpaired t-test). Maximum responses to sodium nitroprusside and glyceryl trinitrate were not achieved (Figure 1c,d). Like MAHMA NONOate, each of these NO donor drugs was less potent on platelets than on pulmonary artery, but the relative potencies (pulmonary artery : platelets) ranged from approximately one order of magnitude for GSNO to greater than three orders of magnitude for sodium nitroprusside and glyceryl trinitrate (Table 1). In view of the low potencies of sodium nitroprusside and glyceryl trinitrate on platelets these two NO donor drugs were not examined further.

Mechanisms of inhibition of platelet aggregation

Effects of the guanylate cyclase inhibitor, ODQ ODQ (10 μM) caused significant, though small, parallel shifts in the curves to MAHMA NONOate, whether aggregation was

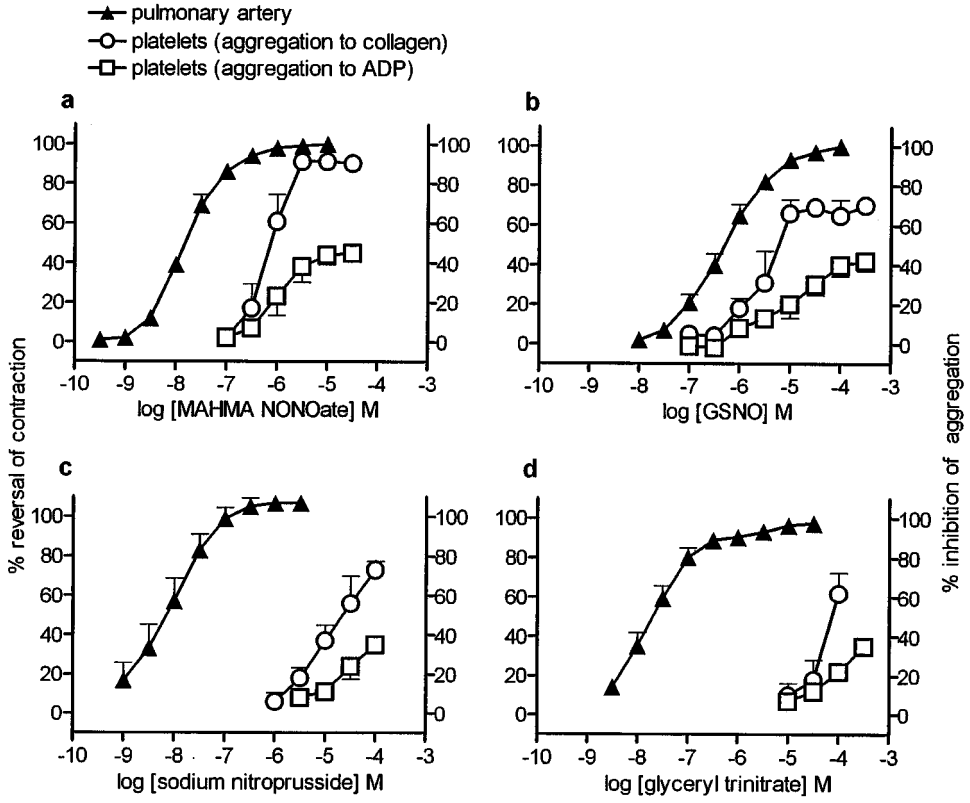

Figure 1 Mean concentration-response curves to (a) MAHMA NONOate, (b) GSNO (S-nitrosoglutathione), (c) sodium nitroprusside and (d) glyceryl trinitrate on rat platelets aggregated sub-maximally with collagen ($2-5$ μg ml^{-1}) or ADP (2 μM) and on rat main pulmonary arteries pre-contracted with phenylephrine (0.1 μM). Responses in platelets are expressed as percentage of inhibition of the aggregation response obtained in the absence of NO donor drug. Relaxation responses in pulmonary artery are expressed as percentage of reversal of the phenylephrine-induced contraction. Points are mean values with s.e.mean shown by vertical bars except when smaller than the size of the symbols. ($n=4-6$).

Table 1 Potencies (negative log IC_{50}) of NO donor drugs as relaxants of pulmonary artery and inhibitors of platelet aggregation

Drug	*Negative log IC_{50}*		*Relative potency[c] (artery : platelets)*
	Artery[a]	*Platelets[b]*	
MAHMA NONOate	7.81±0.08 (4)	6.16±0.14*** (5)	45
GSNO	6.31±0.12 (4)	5.17±0.20** (6)	14
Sodium nitroprusside	8.15±0.22 (4)	4.70±0.18*** (4)	2818
Glyceryl trinitrate	7.71±0.14 (4)	⩽4.21±0.08#*** (4)	⩾3162#

Values are means±s.e.mean. Number of preparations are in parentheses. [a]Pulmonary artery pre-contracted with phenylephrine (0.1 μM). [b]Platelets aggregated with collagen (2–5 μg ml^{-1}). [c]Relative potency=antilog (negative log IC_{50} (pulmonary artery))−(negative log IC_{50} (platelets)). #50% 'inhibition' was not achieved in every experiment, hence the values represent the maximum and minimum possible values of negative log IC_{50} and relative potency, respectively. **$0.01>P>0.001$, ***$P<0.001$ when compared with corresponding values obtained on rat pulmonary artery (unpaired t-test).

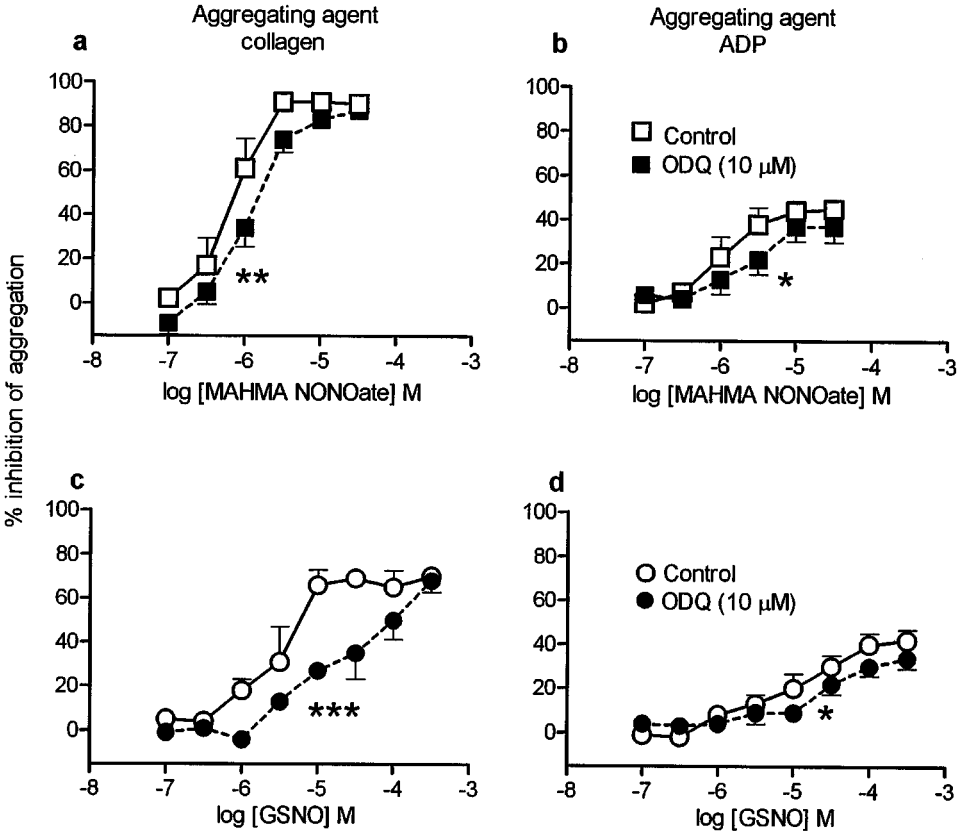

Figure 2 Mean concentration-response curves to (a,b) MAHMA NONOate and (c,d) GSNO (S-nitrosoglutathione) on rat platelets aggregated sub-maximally with collagen (2–5 μg ml^{-1}) or ADP (2 μM) in the absence (control) or presence of ODQ (10 μM). Responses to the NO donor drugs are expressed as percentage of inhibition of the aggregation response obtained in the absence of NO donor drug. Points are mean values with s.e.mean shown by vertical bars except when smaller than the size of the symbols. ($n=5–6$). Note that the control concentration-response curves are the same as those shown in Figure 1. *$0.05>P>0.01$, **$0.01>P>0.001$, ***$P<0.001$ when compared with corresponding concentration-response curve in the absence of ODQ (two-way ANOVA).

induced by collagen (Figure 2a) or by ADP (Figure 2b). To confirm that the effects of 10 μM ODQ represented maximal inhibition of MAHMA NONOate, further experiments were carried out in which 1, 3, 10 and 30 μM ODQ were tested against a single concentration of MAHMA NONOate (3 μM). The inhibition by ODQ was concentration-dependent and 30 μM gave no greater inhibition than 10 μM (Figure 3).

ODQ (10 μM) also inhibited responses to GSNO (Figure 2c,d). However, it was noted that when collagen was the aggregating agent the shift in the GSNO curve was more

pronounced than the corresponding shift obtained with MAHMA NONOate (compare Figure 2c with Figure 2a). This was particularly obvious at concentrations of GSNO >10 μM and, consequently, the curve to GSNO in the presence of ODQ appeared biphasic (Figure 2c).

It is important to note that in the presence of 10 μM ODQ pronounced responses to MAHMA NONOate and GSNO remained (Figure 2).

Effects of the NO-independent activator of soluble guanylate cyclase, YC-1, and the cGMP analogue, 8-pCPT-cGMP YC-1 at concentrations up to 100 μM (the highest concentration that could be studied because of the effect of the vehicle, DMSO) had no effect on collagen-induced aggregation but caused concentration-dependent relaxation of endothelium-denuded pulmonary artery (Figure 4a). In one further platelet aggregation experiment, with 100 μM YC-1, the incubation time was increased from 3 to 10 min but still no inhibition of aggregation occurred.

8-pCPT-cGMP inhibited platelet aggregation (effects on collagen > effects on ADP) but only at concentrations $\geqslant 300$ μM (Figure 4b). It was much less potent on platelets (collagen-induced aggregation, IC_{50} 3.19 ± 0.04; $n=3$) than on pulmonary artery (IC_{50} 5.17 ± 0.05; $n=4$: Figure 4b: $P<0.001$; unpaired t-test).

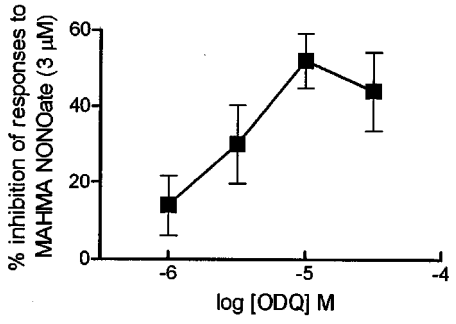

Figure 3 Mean inhibition of responses to MAHMA NONOate (3 μM) by ODQ (1, 3, 10 and 30 μM) on rat platelets aggregated sub-maximally with ADP (2 μM). Responses are expressed as percentage of inhibition of the control response to MAHMA NONOate. Points are mean values with s.e.mean shown by vertical lines. ($n=5-6$).

Effects of the SERCA inhibitor, thapsigargin (in the presence of ODQ) Thapsigargin was examined in the presence of ODQ (10 μM) in order to determine its effects on the ODQ-resistant component of the response to MAHMA NONOate. Concentrations of MAHMA NONOate that gave sub-maximal responses, in the presence of ODQ, were chosen for these experiments. Two concentrations (1 and 3 μM) were examined in the experiments in which collagen was the aggregating agent, but only one concentration (3 μM) in the experiments with ADP, due to the limited response range to MAHMA NONOate with this aggregating agent (Figure 2a,b). Thapsigargin significantly reduced the responses to MAHMA NONOate regardless of the aggregating agent (Figure 5a,b). In the experiments with collagen, responses to MAHMA NONOate were almost abolished by thapsigargin (Figure 5a). In contrast, in the experiments with ADP, more than half of the response to MAHMA NONOate remained when thapsigargin was present (Figure 5b).

Thapsigargin had comparable effects on the ODQ-resistant responses to GSNO (Figure 5c,d). In collagen-aggregated platelets responses to 10 and 100 μM GSNO were almost abolished in the presence of thapsigargin (Figure 5c). In ADP-aggregated platelets, thapsigargin caused significant inhibition, but nevertheless almost half of the response to GSNO (100 μM) still remained (Figure 5d).

It was noted that in the presence of thapsigargin (200 nM) there was a small increase in the size and duration of the aggregation response to both collagen and ADP. However, in a series of control experiments it was ascertained that this augmented aggregation could not account for the reduction by thapsigargin of responses to the NO donors. In these control experiments instead of adding thapsigargin, the concentrations of collagen or ADP were raised. The higher concentration of collagen and ADP caused increases in the aggregation responses similar to those caused by thapsigargin but did not inhibit responses to the NO donors. Under these conditions the responses to 3 μM MAHMA NONOate were: (percentage of aggregation; $n=4$) collagen 47 ± 10.0 and ADP

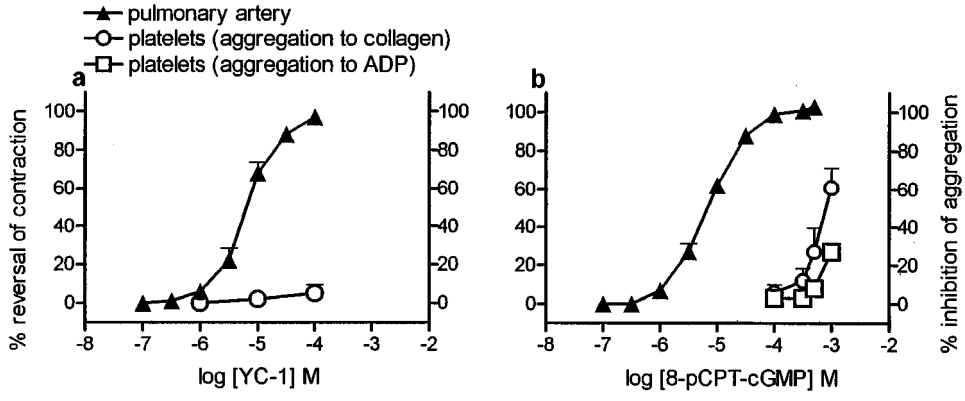

Figure 4 Mean concentration-response curves to (a) YC-1, a soluble guanylate cyclase activator, and (b) 8-pCPT-cGMP, a cGMP analogue, on rat platelets aggregated sub-maximally with collagen (5 μg ml^{-1}; $n=3-4$) or ADP (2 μM; $n=4$) and on rat main pulmonary arteries pre-contracted with phenylephrine (0.1 μM; $n=4$). Responses in platelets are expressed as percentage of inhibition of the aggregation response obtained in the absence of NO donor drug. Relaxation responses in pulmonary artery are expressed as percentage of reversal of the phenylephrine-induced contraction. Points are mean values with s.e.mean shown by vertical bars except when smaller than the size of the symbols.

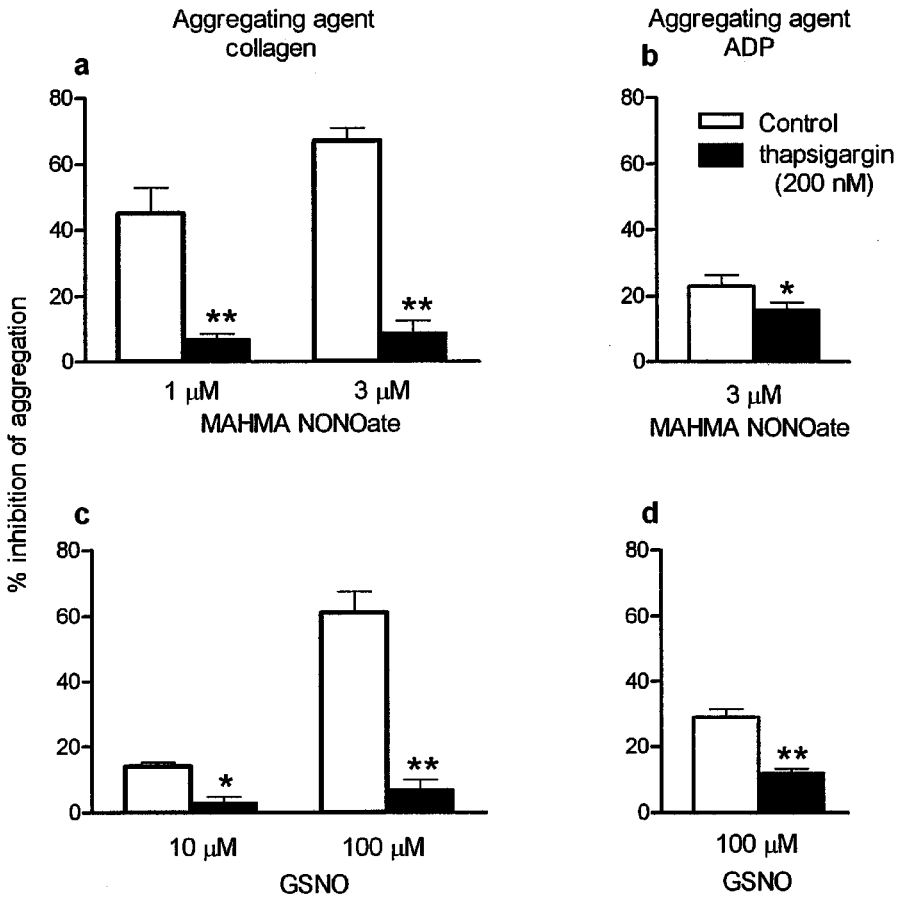

Figure 5 Mean inhibitory responses to (a,b) MAHMA NONOate (1 and 3 μM) and (c,d) GSNO (S-nitrosoglutathione; 10 and 100 μM) on rat platelets aggregated sub-maximally with (a,c) collagen (2–5 μg ml^{-1}) or (b,d) ADP (2 μM) in the absence (control) or presence of thapsigargin (200 nM). ODQ (10 μM) present throughout. Responses to the NO donor drugs are expressed as percentage of inhibition of the aggregation response obtained in the absence of NO donor drug. Points are mean values with s.e.mean shown by vertical lines. ($n=4-8$). *$0.05>P>0.01$, **$0.01>P>0.001$ when compared with corresponding control values in the absence of thapsigargin (paired t-test).

28 ± 1.9; responses to 100 μM GSNO were collagen 46 ± 9.7 and ADP 29 ± 3.7. These values were not significantly lower than the corresponding values for MAHMA NONOate and GSNO obtained in the absence of thapsigargin shown in Figure 5 ($P>0.05$, unpaired t-test).

Effect of superoxide dismutase and catalase The presence of superoxide dismutase (150 IU ml^{-1}) plus catalase (1200 IU ml^{-1}) had no effect on the concentration-response curves to MAHMA NONOate on platelets aggregated with collagen ($n=3$; data not shown). Superoxide dismutase and catalase were not examined on ADP-aggregated platelets (for reasons see Discussion).

Discussion

This is the first study to characterize the inhibition by MAHMA NONOate of platelet aggregation in rats. The inhibitory effect of MAHMA NONOate was shown to be due to the NO component of the drug because the nucleophile, MAHMA, had no effect on rat platelet aggregation. Most of the response to MAHMA NONOate was resistant to the selective soluble guanylate cyclase inhibitor, ODQ, indicating that it occurs independently of the soluble guanylate cyclase/cGMP pathway. This was true whether platelets were aggregated with collagen or ADP. The ODQ-resistant component of the response was blocked by the SERCA inhibitor thapsigargin, suggesting that activation of SERCA may contribute to the response. These data are in contrast to previous findings on rat pulmonary artery, where the main mechanism of vasorelaxation by NONOates is *via* activation of soluble guanylate cyclase and ODQ-resistant mechanisms make only a minor contribution (Homer & Wanstall, 2000).

MAHMA NONOate was less effective as an inhibitor of platelet aggregation than as a vasorelaxant. The same was shown for each of the other NO donors examined. The potency differences between the two tissues were comparable for MAHMA NONOate and GSNO, i.e. less than two orders of magnitude. In contrast, for glyceryl trinitrate and sodium nitroprusside (both of which require the presence of tissue to generate NO) the potency difference was much more pronounced, i.e. both drugs were very poor inhibitors of platelet aggregation. For glyceryl trinitrate this finding is consistent with earlier reports that platelets lack the necessary tissue metabolizing enzymes required for bioactivation of this organic nitrate (Aissa & Feelisch, 1992; Weber *et al*., 1996; Feelisch, 1998). For sodium nitroprusside, the effect on rat

platelets was poor not only compared with that of MAHMA NONOate or GSNO but also compared with its effect on human platelets (Amano *et al*., 1994; Negrescu *et al*., 1995; Sogo *et al*., 2000a). The reason for the poor potency of sodium nitroprusside on rat platelets has not been elucidated but it is possible that rat platelets, unlike human platelets, lack the particular enzymes and/or reducing agents that are allegedly required for bioactivation of this particular NO donor (Kowaluk *et al*., 1992; Rochelle *et al*., 1994; Ku, 1996).

The effectiveness of each of the NO donor drugs examined in this study was consistently dependent on the aggregating agent used, i.e. they were all more effective at inhibiting collagen than ADP. In the experiments with MAHMA NONOate and GSNO, where maximum responses were achieved, it was shown that the main difference between the collagen and ADP experiments was a difference in maximum. For these drugs maximally effective concentrations caused only partial inhibition of ADP. There are other reports where the effects of various NO donor drugs on different aggregating agents are not identical. For example, various sydnonimines and organic nitrates were shown to have different potencies against ADP, collagen and also thrombin but, in that particular study, the maximum responses were not provided (Ivanova *et al*., 1993). In another study, sodium nitroprusside was reported to be effective against platelet activating factor and adrenaline but not against ADP (Parrish & Larson, 1999). In contrast, sodium nitroprusside, two S-nitrosothiols and a NONOate were found to be equieffective against both collagen and ADP in human platelets (Sogo *et al*., 2000a).

The reason for the more marked effect against collagen than against ADP in the present study needs to be considered. It could suggest that the mechanisms of aggregation for ADP and collagen are not identical, and there is evidence in the literature to support this view. For instance, it has been demonstrated that during aggregation to collagen, but not ADP, superoxide anion is generated (Caccese *et al*., 2000). Superoxide anion interacts with NO to form peroxynitrite, which is, itself, an effective inhibitor of platelet aggregation (Yin *et al*., 1995). Hence the greater inhibition of collagen-induced aggregation by the NO donor drugs might theoretically reflect the formation of peroxynitrite. However, data obtained in the present study excluded this possibility because superoxide dismutase and catalase, used to remove superoxide and prevent the formation of peroxynitrite, did not alter responses to MAHMA NONOate when tested against collagen. Another difference between collagen and ADP-induced aggregation is that ADP is the only platelet agonist known to cause a fast, transient, calcium entry that is mediated by a receptor-operated cation channel (Geiger *et al*., 1992). It is tempting to speculate that activation of this channel may be responsible for the difference between inhibition of aggregation to collagen and ADP, particularly as calcium influx *via* the receptor-operated channel has been shown to be insensitive to the NO donor, sodium nitroprusside (Geiger *et al*., 1992; Sage *et al*., 2000). The precise role of receptor-operated channels in aggregation to ADP has not been fully elucidated but it has recently been suggested that the rapid calcium influx through these channels potentiates the effects of ADP-stimulated inositol 1,4,5-triphosphate (Sage *et al*., 2000). This might conceivably render the aggregation response more resistant to the anti-aggregatory effects of NO donors.

One of the aims of this study was to investigate the mechanisms of inhibition of platelet aggregation by MAHMA NONOate, bearing in mind that the effects of NO donor drugs on aggregation are not necessarily identical to their effects on changes in intracellular calcium (Ivanova *et al*., 1993). In studying the mechanism of action of MAHMA NONOate, the soluble guanylate cyclase inhibitor, ODQ (Garthwaite *et al*., 1995), was used as a tool to investigate the contribution made by the soluble guanylate cyclase/cGMP pathway to the inhibitory responses on rat platelet aggregation. Inhibition by MAHMA NONOate, whether induced by collagen or ADP, was largely ODQ-resistant. These results differ quantitatively from the effects of ODQ on vasorelaxation responses to MAHMA NONOate in rat pulmonary artery (Homer *et al*., 1999). The approximate shifts by ODQ of the concentration-response curves to MAHMA NONOate were half a log unit on platelets (present study) compared with two log units on pulmonary artery (Homer *et al*., 1999). Importantly, in both tissues it was shown that no further inhibition could be achieved by raising the concentration of ODQ to 30 μM (Homer & Wanstall, 2000; present study), confirming that maximal inhibition of responses to MAHMA NONOate had been achieved with 10 μM ODQ. These findings indicate that activation of the soluble guanylate cyclase/cGMP pathway is of very little importance in responses to MAHMA NONOate on rat platelets, and certainly less important than on rat pulmonary artery. These results on platelets are of particular significance in relation to the use of NO donor drugs as experimental tools, as it has been the conventional view, and often assumption, that NO donors act via cGMP in this tissue (Heemskerk *et al*., 1994).

There are three possible explanations for the minimal involvement of soluble guanylate cyclase/cGMP pathway in the inhibition by MAHMA NONOate of aggregation in rat platelets. These are (i) that there is little or no soluble guanylate cyclase present in this particular tissue, (ii) that soluble guanylate cyclase is present but is haeme-deficient in rat platelets as suggested by Severina & Busygina (1991), with the small block by ODQ reflecting inhibition of haeme-independent activation of the enzyme, or (iii) that cGMP is not an effective inhibitor of aggregation. There is recent evidence that both NO donor drugs and the NO-independent activator of soluble guanylate cyclase, YC-1, can increase cGMP levels (up to 20 fold above basal levels) in rat platelets (Becker *et al*., 2000). This finding of Becker *et al*. (2000) excludes the first two possible explanations because an increase in cGMP indicates the presence of soluble guanylate cyclase and YC-1 is only able to activate the enzyme if it contains haeme (Friebe *et al*., 1996). Futhermore, activation of non-haeme sites produces only small increases in cGMP (Ignarro *et al*., 1982; Tseng *et al*., 2000) and our data with the cGMP analogue suggest that cGMP has an effect only at very high concentrations. Hence, the most likely explanation for the very limited block by ODQ is the third alternative, namely that cGMP is not an effective inhibitor of aggregation in rat platelets. This is consistent with our cGMP analogue data (very low potency) and compatible with our data for YC-1 (no effect).

Since soluble guanylate cyclase/cGMP was found to be of little importance in responses to MAHMA NONOate in rat platelets, it raised the question of what other mechanism(s) contribute to the response. A likely candidate was stimulation of calcium uptake into intracellular stores following activation of SERCA. It was shown, as early as 1994, that a NO donor drug could accelerate the re-uptake of calcium into intracellular stores in platelets (Doni *et al*., 1994). In a recent study in human platelets it was specifically shown that the inhibitory effect of NO gas on elevations in intracellular calcium involved activation of SERCA and this effect was found to be largely independent of cGMP, i.e. it was seen in the presence of ODQ (Trepakova *et al*., 1999). To test whether this mechanism applies to the inhibition by MAHMA NONOate of aggregation (the functional response examined in the present study) the selective inhibitor of SERCA, thapsigargin, was used. Responses to MAHMA NONOate, in the presence of ODQ, were inhibited, or even abolished, by thapsigargin.

It could possibly be argued that the effect of thapsigargin simply reflects an increase in intracellular calcium, causing functional antagonism of the responses to MAHMA NONOate. However, two observations in the present study are inconsistent with this argument. First, in control experiments, in which the concentrations of collagen and ADP were raised to give responses comparable to those in the presence of thapsigargin, no significant inhibition of the effects of MAHMA NONOate was observed. This argues against functional antagonism because increasing the concentrations of collagen and ADP would, presumably, lead to increases in the concentration of intracellular calcium; yet this procedure had no effect on the response to the NO donor drug. The second observation that argues against functional antagonism is that thapsigargin was not equally effective in the collagen and ADP experiments. When collagen was the aggregating agent thapsigargin virtually abolished the responses to MAHMA NONOate but, in contrast, when ADP was the aggregating agent, small inhibitory responses consistently remained in the presence of both ODQ and thapsigargin. If thapsigargin were simply acting as a functional antagonist one would not predict these differences. Therefore we conclude that the most likely explanation for the results with thapsigargin is that NO, generated by MAHMA NONOate, causes specific activation of SERCA.

It is not known what mechanism is involved in the thapsigargin-resistant component of the response to MAHMA NONOate when ADP is the aggregating agent. Hypothetically it could be a direct inhibitory effect of NO, or a related NO species, on the ADP receptor as suggested by Sogo *et al*. (2000a). They have proposed that NO generated outside the cell from NONOates is likely to be oxidized to produce NO species capable of nitrosatation and nitration of target molecules on the cell surface, such as the ADP receptor, but this remains speculative.

For the most part the results with GSNO were the same as those obtained with MAHMA NONOate. However, when collagen was the aggregating agent one unexpected difference was noted. The magnitude of the block of GSNO by ODQ depended on the concentration of GSNO. The block was most pronounced at concentrations $>10\ \mu$M, where it was greater than that seen for MAHMA NONOate. Thus at higher concentrations of GSNO, activation of soluble guanylate cyclase may assume greater importance. We have previously noted that activation of soluble guanylate cyclase is more important when NO is generated from NO donors following tissue activation than when it is generated 'spontaneously' (Homer *et al*., 1999). GSNO can generate NO in platelet poor plasma but the rate of generation is increased when platelets are present (Megson *et al*., 2000). This suggests that NO generation from GSNO occurs both 'spontaneously' and also following tissue bioactivation. Perhaps tissue-dependent generation of NO assumes greater importance at higher concentrations of GSNO.

In conclusion, there are three key findings in this study. First, MAHMA NONOate inhibited rat platelet aggregation and, like GSNO, the potency difference between platelets and blood vessels was far less pronounced than that for glyceryl trinitrate and sodium nitroprusside. Second, the inhibitory responses were almost completely resistant to the effects of the soluble guanylate cyclase inhibitor, ODQ, suggesting that activation of soluble guanylate cyclase is relatively unimportant in relation to the anti-aggregotory effects of MAHMA NONOate in rat platelets. Because this contrasts with blood vessels, where activation of this enzyme is important, this could explain, at least in part, the potency difference between the two tissues. Third, in this functional study we have obtained data indicating that inhibition of platelet aggregation by MAHMA NONOate probably involves soluble guanylate cyclase-independent activation of SERCA. This latter result extends the findings of Trepakova *et al*. (1999) who examined the effects of NO gas in a biochemical study measuring intracellular calcium. In the present study aggregation was the only functional response examined, and the contribution that NO activation of soluble guanylate cyclase and SERCA may make to inhibition of platelet adhesion and degranulation by MAHMA NONOate remains to be elucidated. One possible corollary of these findings is that should any component of the soluble guanylate cyclase/cGMP pathway be down-regulated in a disease state, vasorelaxation would be affected more than platelet inhibition, and hence the potency difference between these two responses could be diminished.

We gratefully acknowledge support by The National Health and Medical Research Council of Australia. J.C. Wanstall is a NH & MRC Senior Research Fellow. We also acknowledge the excellent technical advice and support provided by A. Gambino.

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(Received August 28, 2002
Accepted September 9, 2002)