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Effects of guanylyl cyclase and protein kinase G inhibitors on vasodilatation in non-tolerant and tolerant bovine coronary arteries

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Abstract

The effects in bovine coronary arteries of the soluble guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) were examined in order to establish the relative importance of the enzyme (a) in the vasodilator actions of glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine and (b) in induction of tolerance to these agents. ODQ strongly inhibited responses to both relaxants with IC_{50}'s of the order of 0.5 μM; in contrast, the protein kinase G inhibitor, 8-bromoguanosine-3′,5′-monophosphorothioate (Rp-8-Br-cGMPS) had little effect on the responses. Tolerance after pre-incubation with glyceryl trinitrate (10 μM) was unaffected by co-pre-incubation with ODQ (1.0 μM), but similar experiments with *S*-nitroso-*N*-acetylpenicillamine were inconclusive because tolerance was associated with depressed contractile activity. It is concluded that in bovine coronary arteries soluble guanylyl cyclase is essential for vasorelaxation to both glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine but is unimportant for induction of tolerance to glyceryl trinitrate. Our results add weight to the hypothesis of impaired biotransformation rather than guanylyl cyclase desensitisation as the mechanism of in vitro nitrate tolerance.

Keywords: Coronary artery, bovine; Guanylyl cyclase soluble; Protein kinase G; Glyceryl trinitrate; *S*-Nitroso-*N*-acetylpenicillamine; Tolerance

1. Introduction

The vasodilator action of glyceryl trinitrate in coronary vasculature is characterised by loss of efficacy during sustained application (tolerance) and low efficacy in small resistance type arteries compared with large epicardial arteries (heterogeneity). Deficient bioconversion to nitric oxide has been implicated in both actions, largely on the basis of experiments on isolated arteries where the effects of glyceryl trinitrate have been compared with those of nitric oxide donors which do not utilise the glyceryl trinitrate metabolising system for nitric oxide generation. Interpretation of the results has generally been based on an assumption that the sequence of events which follow nitric oxide generation and result in relaxation, is common to all nitric oxide-donors. In the present study, we have tested this assumption by examining the effects of an inhibitor of soluble guanylyl cyclase and of an inhibitor of protein kinase G on responses and tolerance induction to glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine in bovine isolated coronary arteries. The inhibitors comprise, for soluble guanylyl cyclase, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (Garthwaite et al., 1995) and for protein kinase G, 8-bromoguanosine-3′,5′-monophosphorothioate, Rp-isomer (Rp-8-Br-cGMPS) (Butt et al., 1990; Zhuo et al., 1994). *S*-Nitroso-*N*-acetylpenicillamine was selected as an nitric oxide donor since its actions appear to be the most sharply distinguished from those of glyceryl trinitrate in earlier comparative studies on bovine coronary arteries, as illustrated by minimal cross-tolerance in glyceryl trinitrate tolerant arteries (Henry et al., 1989a,b) and by absence of heterogeneity (De la Lande et al., 1996b).

The study was prompted by reports of partial dissociation and even independence of relaxation from cGMP formation on the part of some nitric oxide donors. The nitric oxide donors include nitric oxide itself, *S*-nitroso-*N*-acetylpenicillamine and *S*-nitrosoglutathione and the vessels include rat aorta, rabbit aorta and bovine pulmonary artery (Kowaluk and Fung, 1990; Bolotina et al., 1994; Brunner et al., 1996). In addition, a dissociation at the

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level of protein kinase G activation is suggested by failure of a protein kinase G inhibitor to affect the relaxant action of *S*-nitroso-*N*-acetylpenicillamine in the rat aorta while abolishing relaxation to glyceryl trinitrate (Brooks and Majewski, 1995; Van der Zypp and Majewski, 1998).

2. Materials and methods

2.1. Materials

Bradykinin acetate, (−)-isoprenaline HCl and U46619 (9,11-dideoxy-11*a*,9*a*-epoxy-methano-prostaglandin F_{2a}) were purchased from Sigma, St. Louis, MO, USA. Glyceryl trinitrate was purchased from Fisons, Australia and *S*-nitroso-*N*-acetylpenicillamine from Colour your Enzyme, Bath, Ontario, Canada. ODQ was purchased from Tocris Cookson, Langford, Bristol, UK and 8-pCPT-cGMP (8-[4-chlorophenylthio]guanosine-3′,5′-monophosphate) and Rp-8-Br-cGMPS were purchased from BioLog Life Science Institute, Bremen, Germany. Stock solutions were made up in either ethanol (glyceryl trinitrate, U46619, *S*-nitroso-*N*-acetylpenicillamine), distilled water (bradykinin, 8-pCPT-cGMP, isoprenaline, Rp-8-Br-cGMPS) or dimethyl sulfoxide (ODQ) and stored at −20°C. Dilutions of the stock solutions were made in distilled water and maintained on ice.

The Krebs solution was gassed with carbogen (95% O_2, 5% CO_2) and was of the following composition (mM): NaCl (118), KCl (3.89), KH_2PO_4 (1.18), $NaHCO_3$ (25), $MgCl_2$ (1.05), $CaCl_2$ (2.34), EDTA (0.01) and glucose (5.56), pH7.4. High K^+ solution, referred to as potassium physiological salt solution (KPSS), was obtained by replacing the NaCl in Krebs solution with iso-osmolar KCl.

2.2. Artery preparation

Fresh bovine heart in ice-cold Krebs solution was transported to the laboratory within 20 min. Rings (3 mm length) were dissected from the middle region of the left anterior descending artery and mounted in organ baths each containing 15 ml of Krebs solution at 37°C. Isometric tension was recorded via two stainless steel wires through the lumen, one of which was fixed and the other attached to a Grass FT03 transducer. The baseline tension was maintained at the mean (4.5 g) of normalised values determined in earlier study (De la Lande et al., 1996b).

2.3. Experimental procedure

In all experiments after the baseline tension was stable, (usually within 1 h), the artery was contracted successively with KPSS (to assess viability) and with U44619 (0.1–0.3 μM). Prior to washing out the U46619, bradykinin (0.1–0.2 μM) was added to assess endothelium function; if the relaxant response was less than 50%, the artery was discarded. In some experiments, the endothelium was removed by dental floss wrapped around a glass capillary tube. Relaxation in response to bradykinin of less than 10% was used as the index of effective removal.

2.3.1. Responses to vasorelaxants

The vasorelaxants were glyceryl trinitrate, *S*-nitroso-*N*-acetylpenicillamine, 8-pCPT-cGMP, isoprenaline and bradykinin. The artery was contracted to a steady state sub-maximal level with U44619 (0.01–0.03 μM) after which the relaxant was applied cumulatively at half log intervals of concentration in order to define its concentration–response curve. Approximately 60 min after repeated washout of the relaxant, a second contractile response to KPSS was elicited in order to quantitate the level of submaximal contraction to U46619. In the present experiments, the responses to the U46619 were 81 + 1% ($n = 49$) of those to the second KPSS. The use of the response to KPSS as a measure of the maximal response to U46619 is based on results of earlier experiments in which the response to KPSS was followed by the response to a high supramaximal concentration (3 μM) of U46619. The paired comparison ($n = 7$) indicated that the two responses did not differ significantly.

2.3.2. Effect of guanylyl cyclase inhibition

The inhibitor (ODQ) was added 30 min before the relaxant. In some experiments, ODQ was added only when the U46619 contracted artery had been fully relaxed by 10 μM glyceryl trinitrate or *S*-nitroso-*N*-acetylpenicillamine, after which the recovery of contractile tone was monitored; a second fully relaxed artery ring which had not received ODQ served as the control.

2.3.3. Effect of protein kinase G inhibition

The inhibitor (Rp-8-Br-cGMPS) was added 60 min prior to the relaxant. Note: In these experiments and also in those where the protein kinase G agonist, 8-pCPT-cGMP was used, the experimental procedures were modified in two ways, namely (a) the organ bath volume was reduced to 1.5 ml to take into account the high cost of the agents and (b) only endothelium-denuded arteries were used in order to mimic the conditions used by Brooks and Majewski (1995) in rat aorta.

2.3.4. Effect of ODQ on induction of tolerance to glyceryl trinitrate and S-nitroso-N-acetylpenicillamine

Four artery rings were treated as follows.

Ring 1: The ring was pre-incubated with ODQ (1.0 μM) for 60 min and with the relaxant (10 μM) for the last 40 min of this period. Sixty minutes after repeated washout, the concentration–response curve to the relaxant was elicited as described above. In the remaining rings, the pre-incubation procedure was modified as follows.

Ring 2: ODQ was omitted and replaced by its vehicle (7 mM dimethyl sulfoxide).

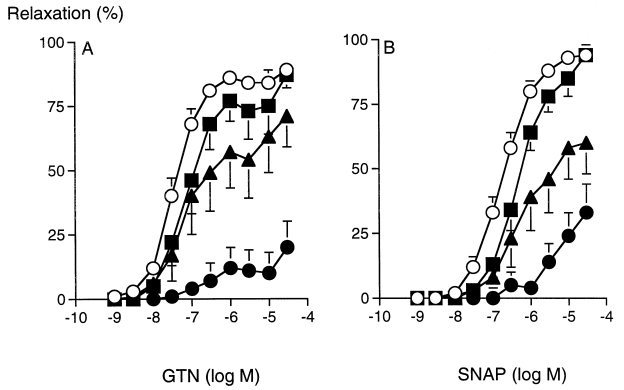

Fig. 1. Effect of ODQ on relaxant responses to glyceryl trinitrate (GTN; A) and *S*-nitroso-*N*-acetylpenicillamine (SNAP; B) in arteries pre-incubated with vehicle only (○, $n = 18$) and with ODQ (■, 0.1 μM; ▲, 0.3 μM; ●, 1.0 μM), $n = 5–8$.

Ring 3: The relaxant was omitted and replaced by its vehicle (8 mM ethanol).

Ring 4: Both ODQ and relaxant were omitted and replaced by their vehicles.

Note: The experimental conditions represented a compromise between (a) adequate reversibility of ODQ's inhibitory action; hence the use of a 1.0 μM concentration and the long washout time of 60 min and (b) a concentration of glyceryl trinitrate or *S*-nitroso-*N*-acetylpenicillamine (10 μM) high enough to maximise detection of tolerance after 60 min washout.

2.3.5. cGMP formation

Artery rings were equilibrated for 60 min prior to transfer to 5 ml glass tubes containing 2 ml Krebs gassing at 37°C. ODQ (1.0 μM) or vehicle was added 20 min prior to glyceryl trinitrate or *S*-nitroso-*N*-acetylpenicillamine (each 10 μM). Rings were incubated for 1 min, snap frozen in liquid nitrogen and stored at −80°C until assay. cGMP formation was estimated by the method of Tadjkarimi et al. (1992), with minor modifications. In brief, crushed frozen segments were incubated in 1 ml of 6% trichloroacetic acid for 60 min on ice and following centrifugation at $2000 \times g$, for 15 min at 4°C, the pellets were stored at −20°C and used to estimate protein content (Lowry method), while the supernatants were extracted four times with 5 ml of water saturated ether. The aqueous phase was then dried down under a stream of nitrogen at 60°C and the samples reconstituted in assay buffer. cGMP was measured following acetylation of the samples, using a cGMP-^{125}I radioimmunoassay kit (Amersham, UK) and the results were expressed as nmol/mg protein.

2.4. Data analysis

Relaxant responses were expressed as percent decrease in contractile tone. EC_{50} and E_{max} values for relaxants other than glyceryl trinitrate were derived from the relaxant concentration–response curves using the non-linear regression programs for sigmoid curves in Graphpad Prism 2.01 or KaleidaGraph 3.0.1. The concentration–response curve for glyceryl trinitrate was biphasic in shape and the E_{max} and EC_{50} values for this agent refer only to the first phase maximum.

Tolerance to glyceryl trinitrate or *S*-nitroso-*N*-acetylpenicillamine was manifested by rightward shifts and depressed maxima of concentration–response curves. It was quantitated in terms of percent decrease in maximum and by the ratio (tolerant–non-tolerant) in log units of EC_{50}'s. A further quantitation was in terms of the ratio (in log units) of the areas under the log concentration–response curves (non-tolerant–tolerant). The upper limits of concentrations used in calculating the areas under the log concentration–response curves were 1.0 μM for glyceryl trinitrate and 3.0 μM for *S*-nitroso-*N*-acetylpenicillamine; these concentrations are in the approximate ratio of the respective EC_{50}'s in the non-tolerant artery. All results are expressed as either mean ± S.E.M. or mean (± confidence intervals). Significance of differences ($P < 0.05$) was determined by paired or unpaired *t*-test as specified in results. *n* refers to the number of animals.

3. Results

3.1. Effect of ODQ

3.1.1. ODQ and glyceryl trinitrate

The relaxant action of glyceryl trinitrate was characterised by a sigmoid concentration–response curve with a maximum of 87 ± 4% ($n = 18$) in the vicinity of 1.0 μM. In the presence of ODQ, the curve was depressed and biphasic in shape with a first phase maximum in the 1.0–3.0 μM range, followed by further relaxation at 10–30 μM. This biphasic curve shape has previously been seen in glyceryl trinitrate tolerant arteries by Henry et al. (1989b).

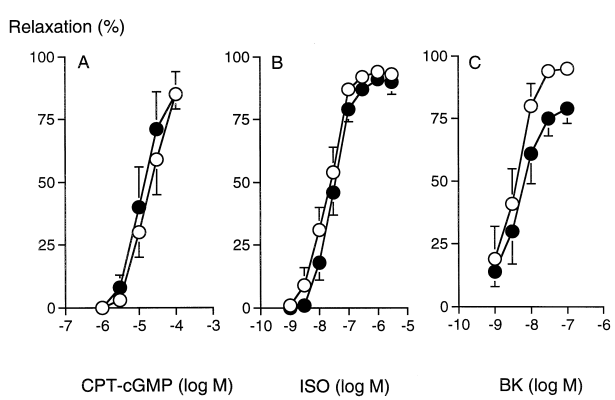

Fig. 2. Effect of ODQ (10 μM) on relaxant responses to 8-pCPT-cGMP (A, $n = 4$), isoprenaline (ISO; B, $n = 5$) and bradykinin (BK; C, $n = 8$) in control (○) and ODQ (●) treated arteries.

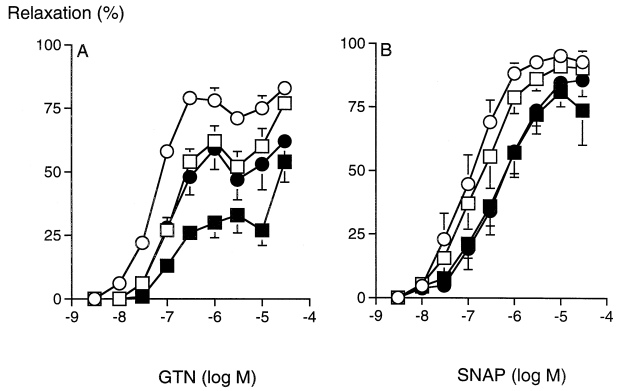

Fig. 3. Effect of pre-incubation with ODQ (1.0 μM) on tolerance to 10 μM glyceryl trinitrate (GTN; A, $n = 8$) and responses to *S*-nitroso-*N*-acetylpenicillamine (SNAP; B, $n = 6–8$) showing absence of cross-tolerance in glyceryl trinitrate tolerant arteries and lack of effect of ODQ, in arteries pre-incubated with vehicle (○), with 1.0 μM ODQ (●), with 10 μM glyceryl trinitrate (□) and with glyceryl trinitrate plus ODQ (■).

As indicated by the concentration–response curves in Fig. 1a, the inhibitory action of ODQ was characterised by a threshold in the vicinity of 0.1 μM and a near maximal effect at 1.0 μM. ODQ 10 μM abolished responses to all concentrations of glyceryl trinitrate ($n = 6$, data not shown). ODQ concentration effect curves based on depression of responses to 10 μM glyceryl trinitrate indicated a mean IC_{50} of about 0.6 μM. Inhibition by ODQ 1.0 μM was substantially, but not completely, reversed by washing for 60 min; data on reversibility are included in the tolerance experiments (Section 3.1.4).

When added to the contracted artery after it was fully relaxed with glyceryl trinitrate (10 μM), ODQ in cumulated concentrations of 0.3,1.0 and 10 μM returned the tone within 10–15 min to steady state levels amounting to 63 ± 13, 92 ± 4 and 105 ± 6%, respectively ($n = 3$) of the contracted level; corresponding spontaneous returns of tone in paired controls (ODQ absent) were 24 ± 6, 30 ± 6 and 35 ± 10% ($n = 3$), respectively. In arteries at basal tone (glyceryl trinitrate and U46619 absent) or contracted with U46619 (glyceryl trinitrate absent), a contractile response to ODQ was consistently observed only at the highest concentration (10 μM); the response was usually of the order of 5–10% of the contractile response to U46619.

3.1.2. ODQ and S-nitroso-N-acetylpenicillamine

The relaxant response to *S*-nitroso-*N*-acetylpenicillamine was characterised by a sigmoid concentration–response curve with an E_{max} of 92 ± 3% ($n = 19$) in the 3–10 μM range. ODQ (0.1–10 μM) inhibited the responses in a concentration dependent fashion (Fig. 1b) which closely resembled its interaction with glyceryl trinitrate (Fig. 1a), including the ability of ODQ 10 μM to abolish all relaxant activity and a mean IC_{50} of about 0.5 μM. Similarly, ODQ (0.3–10 μM), when added to an artery which was fully relaxed by *S*-nitroso-*N*-acetylpenicillamine (10 μM), returned contractile tone close to (at 1.0 μM ODQ) or slightly above (at 10 μM ODQ) the pre-nitrosothiol level.

3.1.3. ODQ and other relaxants

ODQ (10 μM) was without effect on relaxant responses to both the protein kinase G agonist 8-pCPT-cGMP and isoprenaline (Fig. 2a,b). It had a slight inhibitory effect ($P < 0.05$) on relaxation to bradykinin in the maximum range of responses (Fig. 2c).

3.1.4. ODQ and tolerance to glyceryl trinitrate

Tolerance in the group of arteries which were pre-incubated with glyceryl trinitrate (10 μM) alone for 40 min followed by 60 min washout was characterised by both a rightward movement of the glyceryl trinitrate concentration–response curve and a lower first-phase maximum when compared with vehicle controls (Fig. 3a). Responses to the contractile agent, U46616, were unchanged. Arteries which were pre-incubated with ODQ (1.0 μM) plus glyceryl trinitrate (10 μM) exhibited similar changes; arteries which were pre-incubated with ODQ alone were the controls (Fig. 3a). Fig. 3a also illustrates the extent of recovery from ODQ's inhibitory action following 60 min washout. The magnitude of tolerance did not differ significantly between the vessels pre-incubated with and without ODQ, irrespective of whether tolerance was estimated in

Table 1
Effect of pre-incubation with GTN[a] and ODQ[b] on responses to GTN

Agent	E^c_{max} (%)	EC^d_{50} (nM)	EC_{50} ratio (tol/control)	AUC ratio[e] (control/tol)
Vehicle	77 ± 4	47 (32–70)	2.0 (1.4–3.0)	1.8 (1.4–2.2)
GTN	59 ± 5[f]	96 (68–135)[f]		
ODQ	54 ± 7	83 (53–129)	1.9 (1.2–3.3)	2.0 (1.4–2.9)
ODQ/GTN	36 ± 7[f]	160 (84–306)[f]		

[a] 10 μM GTN.
[b] 1.0 μM ODQ.
[c] The E_{max} results are expressed as mean ± S.E.M.
[d] The EC_{50} and ratios are expressed as mean (±95% confidence limits).
[e] AUC: area under log concentration–response curve to GTN 1.0 μM.
[f] Effect of GTN pre-incubation significant, $P < 0.05$, paired *t*-test, $n = 8$.
Abbreviations: GTN, glyceryl trinitrate; tol, tolerant.

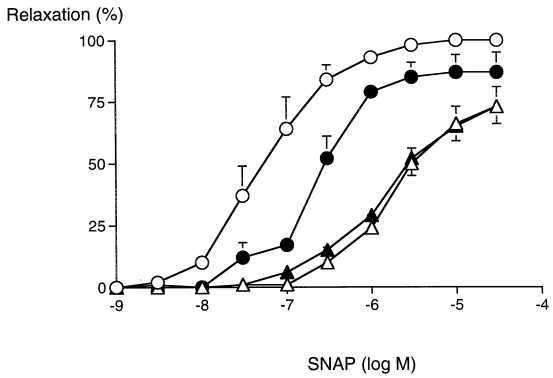

Fig. 4. Effect of pre-incubation with ODQ (1.0 μM) on tolerance to *S*-nitroso-*N*-acetylpenicillamine (SNAP; $n = 8$) in control (○), 10 μM *S*-nitroso-*N*-acetylpenicillamine (△), 1.0 μM ODQ (●) and ODQ plus *S*-nitroso-*N*-acetylpenicillamine (▲) treated arteries.

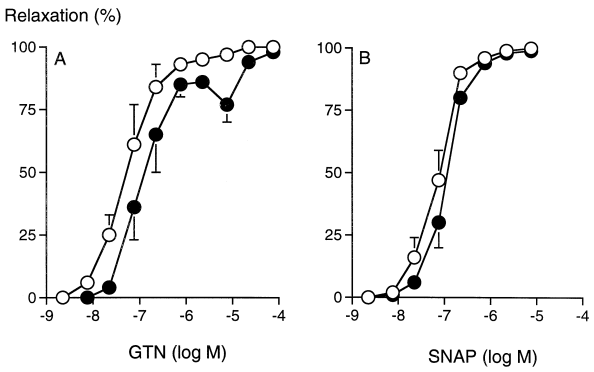

Fig. 5. Effect of Rp-8-Br-cGMPS on relaxant responses to glyceryl trinitrate (GTN; A, $n = 4$) and *S*-nitroso-*N*-acetylpenicillamine (SNAP; B, $n = 5$) in arteries pre-incubated with vehicle only (○) and 450 μM Rp-8-Br- cGMPS (●).

terms of ratios of EC_{50} or areas under concentration–response curves (Table 1). Hence, there was no indication in these experiments that ODQ had interfered with induction of tolerance.

3.1.5. ODQ and cross-tolerance to S-nitroso-N-acetylpenicillamine

Pre-incubation with glyceryl trinitrate did not produce significant changes in sensitivity to *S*-nitroso-*N*-acetylpenicillamine (Fig. 3b), indicating that tolerance to glyceryl trinitrate was not accompanied by cross-tolerance to *S*-nitroso-*N*-acetylpenicillamine. Similarly, vessels pre-incubated with ODQ plus glyceryl trinitrate exhibited the same sensitivity to *S*-nitroso-*N*-acetylpenicillamine as those pre-incubated with ODQ only (Fig. 3b). Conversely, the latter result can be interpreted as evidence that the presence of glyceryl trinitrate during pre-incubation did not influence recovery from ODQ's inhibitory action.

3.1.6. ODQ and tolerance to S-nitroso-N-acetylpenicillamine

Vessels pre-incubated with *S*-nitroso-*N*-acetylpenicillamine were subsensitive to both the *S*-nitrosothiol and the contractile agent, U46619. When ODQ was present during pre-incubation with *S*-nitroso-*N*-acetylpenicillamine, sensitivity to the *S*-nitroso-*N*-acetylpenicillamine was unchanged (Fig. 4) but the depressant effect on contractile activity was much less prominent. The depression of contractile activity (the latter normalised as a percentage ratio of the steady state response to U46619, divided by the initial response to KPSS) after the various pre-incubating agents, was: vehicle only ($124 \pm 9\%$), *S*-nitroso-*N*-acetylpenicillamine only ($73 \pm 10\%$), ODQ only ($131 \pm 12\%$) and ODQ plus *S*-nitroso-*N*-acetylpenicillamine ($102 \pm 8\%$). *S*-Nitrosothiol sensitivity in the control (non-tolerant) vessels was reduced following pre-incubation with ODQ, reflecting incomplete recovery from ODQ's inhibitory action. The net effect was an apparent reduction in the magnitude of tolerance to *S*-nitroso-*N*-acetylpenicillamine (Table 2).

3.1.7. ODQ and cGMP formation

The basal content of cGMP in nmol/mg protein ($n = 7$) was increased during one min from 0.23 ± 0.03 to 8.14 ± 2.06 by glyceryl trinitrate (10 μM) and to 5.84 ± 1.13 by *S*-nitroso-*N*-acetylpenicilamine (10 μM). With ODQ

Table 2
Effect of pre-incubation with SNAP[a] and ODQ[b] on responses to SNAP

Agent	E^{c}_{max} (%)	EC^{d}_{50} (nM)	EC_{50} ratio (tol/control)	AUC ratio[e] (control/tol)
Vehicle	99 ± 1	56 (21–149)	27 (12–59)	6.2 (4.1–9.3)
SNAP	73 ± 8[f]	1503 (543–2094)[f]		
ODQ	89 ± 6	234 (119–475)	5.3 (1.6–1.8)[g]	3.2 (1.6–6.5)[g]
ODQ/SNAP	74 ± 7[f]	1241 (589–2588)[f]		

[a] 10 μM SNAP.
[b] 1.0 μM ODQ.
[c] The E_{max} results are expressed as mean ± S.E.M.
[d] The EC_{50} and ratios are expressed as mean (±95% confidence limits).
[e] AUC: area under log concentration–response curve to 3.0 μM SNAP.
[f] Effect of SNAP pre-incubation significant, $P < 0.05$, paired *t*-test, $n = 8$.
[g] Effect of ODQ pre-incubation significant, $P < 0.05$, paired *t*-test.
Abbreviations: SNAP, *S*-nitroso-*N*-acetylpenicillamine; tol, tolerant.

(1.0 μM) present, the corresponding contents were 0.086 ± 0.019, 1.08 ± 0.73 and 0.49 ± 0.25, respectively. The inhibition of glyceryl trinitrate stimulated and of *S*-nitroso-*N*-acetylpenicillamine stimulated cGMP formation was 86 ± 6 and 87 ± 8%, respectively.

3.2. Effect of Rp-8-Br-cGMPs

The protein kinase G inhibitor Rp-8-BR-cGMPS (450 μM) had a weak inhibitory effect on responses to both glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine (Fig. 5), the respective EC_{50}'s being increased from 53 (13–217) to 109 (31–392) nM ($n = 4$) and 69 (31–153) to 109 (61–195) nM ($n = 5$). The increases were each significant ($P < 0.05$, paired *t*-test).

4. Discussion

The inhibitory interaction between glyceryl trinitrate and ODQ accords both with the close association between the relaxant action of glyceryl trinitrate and cGMP formation in the bovine coronary artery demonstrated by Kukovetz and Holzmann (1986) and with the inhibitory action of ODQ on cGMP formation in other vessels (Garthwaite et al., 1995; Brunner et al., 1996). The latter action was also demonstrated in the bovine coronary artery in the present study. A primary aim of the study was to assess whether the cGMP pathway mediating relaxation was common to the coronary vasodilator actions of glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine. The effects of ODQ reported here suggest that this is the case since in the highest concentration tested (10 μM), it abolished responses to both glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine and at lower concentrations the inhibitory effects on responses to these agents were indistinguishable, irrespective of whether the effect of ODQ was measured by a decrease in relaxation or by restoration of contractile tone after the artery was maximally relaxed. The IC_{50}'s for ODQ with each agent, were in the 0.5–0.6 μM range, which is comparable with 0.3 μM obtained by Garthwaite et al. (1995) for inhibition of purified soluble guanylyl cyclase stimulated by sodium nitroprusside (100 μM) and *S*-nitrosoglutathione (3 μM). The selectivity of ODQ for guanylyl cyclase demonstrated by the latter workers and by Brunner et al. (1996) was indicated in our study by lack of effect on relaxant responses to isoprenaline (cAMP mediated) and to the protein kinase G agonist 8-pCPT-cGMP. The mild inhibitory effect on the relaxant response to bradykinin accords with evidence that this response in the bovine coronary artery is mediated predominantly by the endothelium-derived hyperpolarising factor (Holzmann et al., 1994).

The findings discussed above leave little doubt that cGMP plays a key role in the vasodilator actions of both glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine and, hence, argue against dissociation between these actions and cGMP formation. However, we still cannot exclude the possibility that cellular pathways mediating the relaxant effects of the two agents diverge at some step subsequent to guanylyl cyclase activation. Evidence for divergence based on differential effects of the protein kinase G inhibitor Rp-8-Br-cGMPS on responses to glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine in rat aorta, has been presented recently (Brooks and Majewski, 1995; Van der Zypp and Majewski, 1998). However, the effects of the same inhibitor tested under very similar conditions in our experiments were too marginal to indicate whether similar divergence occurs in the bovine artery.

The failure of ODQ to discriminate between glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine has important implications to theories of heterogeneity and tolerance to glyceryl trinitrate. Thus, the possibility that the low efficacy of glyceryl trinitrate in coronary resistance arteries relative to large conduit arteries is due to differences in soluble guanylyl cyclase activity between the two types of vessel can be excluded on the basis that *S*-nitroso-*N*-acetylpenicillamine is equally active on both types (Sellke et al., 1990; De la Lande et al., 1996b) yet the present findings indicate that differences in soluble guanylyl cyclase activity would have similar effects on responses to the two relaxants. Similarly, the hypothesis that desensitisation of soluble guanylyl cyclase is an important factor in tolerance (Waldman et al., 1986; Rapoport et al., 1987; Romanin and Kukovetz, 1989; Kukovetz and Holzmann, 1990) can be reconciled with evidence of minimum cross-tolerance to *S*-nitroso-*N*-acetylpenicillamine in glyceryl trinitrate-tolerant arteries (Henry et al., 1989b) only if *S*-nitroso-*N*-acetylpenicillamine-mediated relaxation was relatively insensitive to loss of enzyme activity. The effects of ODQ reported here show that this is not the case.

The reversible nature of ODQ's inhibitory action in the bovine coronary artery, seen also in porcine aortic endothelial cells (Brunner et al., 1996), is seemingly at odds with biochemical evidence that inhibition, although competitive for nitric oxide, is irreversible (Schrammel et al., 1996). Nevertheless, the reversibility offered a novel means of assessing the importance of soluble guanylyl cyclase activity in the induction of tolerance to glyceryl trinitrate. The experiments in which vessels were pre-incubated with ODQ and glyceryl trinitrate separately and in combination revealed that ODQ, in a concentration which inhibited cGMP formation by about 90%, had little affect on the magnitude of the tolerance. These results imply that tolerance is induced by a mechanism which is largely independent of soluble guanylyl cyclase activity. Since biotransformation of glyceryl trinitrate to nitric oxide precedes activation of soluble guanylyl cyclase (Kawamoto et al., 1990), the apparent dissociation of tolerance from soluble guanylyl cyclase activity represents new evidence in support of impaired biotransformation (Brien et al., 1986, Henry et al., 1989b) as the mechanism of tolerance. Al-

though there is evidence favouring superoxide-induced inactivation of nitric oxide after its formation as a mechanism of in vivo tolerance (Munzel et al., 1995) the lack of cross-tolerance to *S*-nitrosothiol in our study argues against a similar mechanism for in vitro tolerance in the bovine artery and in this respect accords with the failure of antioxidants to influence in vitro tolerance in rat aorta (Laight et al., 1997).

The absence of cross-tolerance to the *S*-nitrosothiols is important for another reason. Our interpretation of the tolerance data is based on the assumption that the vessels pre-incubated with ODQ only, are valid controls for those pre-incubated with ODQ plus glyceryl trinitrate. The assumption is open to the criticism that in the latter vessels there may have been competitive displacement of ODQ from the enzyme by glyceryl trinitrate during the 40 min pre-incubation period. Such displacement, by hastening recovery from ODQ's inhibitory action during the washout period, would result in an increased relaxant response to glyceryl trinitrate and hence reduce the magnitude of tolerance. However sensitivity to *S*-nitroso-*N*-acetylpenicillamine did not differ between vessels pre-incubated with glyceryl trinitrate plus ODQ and those pre-incubated with ODQ alone (Fig. 3b), i.e., there was no evidence for accelerated recovery of *S*-nitrosothiol sensitivity from ODQ's inhibitory action. Hence, if displacement of ODQ did occur, it was not of sufficient magnitude to invalidate the use of ODQ alone as the control for assessing effects of soluble guanylyl cyclase inhibition on tolerance induction.

The effects of ODQ on tolerance to *S*-nitroso-*N*-acetylpenicillamine were examined under identical conditions to those used in the study of glyceryl trinitrate tolerance. The results confirmed both the marked tolerance to *S*-nitrosothiols reported in earlier studies (Henry et al., 1989a; Zhang et al., 1994) and its association with depression of contractile activity noted for various other *S*-nitrosothiols by Smith et al. (1994). Pre-incubation with ODQ resulted in an apparent reduction in the magnitude of tolerance but the problem arises that ODQ largely restored the level of contraction in the tolerant artery and hence these levels were not identical in the two tolerant groups under comparison. A further complication was that the reduction in tolerance was due largely to the differing *S*-nitrosothiol sensitivities of the two non-tolerant control groups. These problems raise sufficient doubt about the validity of the ODQ controls in these experiments to preclude meaningful interpretation of the relationship between guanylyl cyclase activity and tolerance to *S*-nitroso-*N*-acetylpenicillamine.

In summary, the effects of ODQ in non-tolerant arteries emphasise the key role played by soluble guanylyl cyclase in the vasodilator actions of both glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine and hence add considerable weight to theories of nitrate action based on comparisons between the classes of nitric oxide-donors represented by glyceryl trinitrate and *S*-nitroso-*N*-acetylpenicillamine and which assume a cGMP effector pathway common to both classes. The effects on tolerance induction while inconclusive with respect to the mechanism of S-nitrosothiol tolerance, add strong support to 'impaired biotransformation' rather than 'soluble guanylyl cyclase-desensitisation' as a mechanism of tolerance to glyceryl trinitrate. It is emphasised that the conclusions refer only to the bovine coronary artery and to in vitro tolerance measured after a washout time of 60 min. Lawson et al. (1996) obtained evidence compatible with soluble guanylyl cyclase desensitisation as a mechanism of tolerance in rat aorta but their washout period was considerably shorter than in our experiments. These workers suggest that the mechanism of in vitro glyceryl trinitrate tolerance is multifactorial and the experimental conditions, including periods of exposure and washout may be important determinants of the contribution of soluble guanylyl cyclase desensitisation. Hence, it is conceivable that desensitisation of guanylyl cyclase represents a mechanism from which recovery is too rapid to be detected under our experimental conditions. The type of vessel used may also be important in view of evidence pointing to different factors determining glyceryl trinitrate biotransformation in rat aorta and the bovine coronary artery (De la Lande et al., 1996a).

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