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Carbon monoxide-induced relaxation of the ductus arteriosus in the lamb: evidence against the prime role of guanylyl cyclase

[1]F. Coceani, L. Kelsey & E. Seidlitz

Research Institute, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada

1 We have previously found that carbon monoxide (CO) potently relaxes the lamb ductus arteriosus and have ascribed this response to inhibition of a cytochrome P450-based mono-oxygenase reaction which sustains contractile tone. Our proposal, however, has been questioned on the evidence of findings in other blood vessels implicating the guanylyl cyclase-based relaxing mechanism as the target for CO. To investigate this issue further, we have carried out experiments in the isolated ductus from near-term foetal lambs and have examined the effect of CO concomitantly on muscle tone and cyclic GMP content, both in the absence and presence of guanylyl cyclase inhibitors, or during exposure to monochromatic light at 450 nm.

2 CO (65 μM) reversed completely, or nearly completely, the tone developed by the vessel in the presence of oxygen (30%) and indomethacin (2.8 μM). Cyclic GMP content tended to increase with the relaxation, but the change did not reach significance. Sodium nitroprusside (SNP), a NO donor, mimicked CO in relaxing the ductus. Contrary to CO, however, SNP caused a marked accumulation of cyclic GMP with levels being positively correlated with the relaxation.

3 Methylene blue (10 μM) reduced marginally the CO relaxation, whilst LY-83583 (10 μM) had an obvious, albeit variable, inhibitory effect. Basal cyclic GMP content was lower in tissues treated with either compound and rose upon exposure to CO. However, the levels attained were still within the range of values for tissues prior to any treatment. Furthermore, the elevation in cyclic GMP was not related to the magnitude of the CO relaxation.

4 Illumination of the ductus with monochromatic light at 450 nm reversed the CO relaxation and any concomitant increase in cyclic GMP. In the absence of CO, light by itself had no effect.

5 Ductal preparations with only muscle behaved as the intact preparations in reacting to CO, both in the absence and presence of guanylyl cyclase inhibitors, or during illumination.

6 We conclude that the primary action of CO in the ductus arteriosus is not exerted on the guanylyl cyclase heme and that cyclic GMP may only have an accessory role in the relaxation to this agent. This finding reasserts the importance of a cytochrom P450-based mono-oxygenase reaction for generation of tone and as a target for CO in the ductus.

Keywords: Ductus arteriosus; oxygen; carbon monoxide; nitric oxide; cytochrome P450; guanylyl cyclase/cyclic GMP; photoactivation; methylene blue; LY-83583; nitric oxide synthesis inhibitor

Introduction

It has been known for a long time that CO dilates blood vessels (Coburn *et al.*, 1979; Coceani *et al.*, 1984, 1988; McGrath & Smith, 1984; Vedernikov *et al.*, 1989; Adeagbo *et al.*, 1990; Gräser *et al.*, 1990; Furchgott & Jothianandan, 1991). Only recently, however, has this response gained special attention, and this has followed the realization that the agent may be formed naturally and, like NO, may function as a diffusible regulator of vascular tone (Marks *et al.*, 1991; Ewing *et al.*, 1994; Cook *et al.*, 1995; Johnson *et al.*, 1995). Views differ on the mechanism of action of CO, though there is agreement in excluding interference with the energy supply to the muscle as the cause for this vasodilatation (Coburn *et al.*, 1979; McGrath & Smith, 1984; Coceani *et al.*, 1989). According to many investigators (Brüne *et al.*, 1990; Gräser *et al.*, 1990; Furchgott & Jothianandan, 1991; Ewing *et al.*, 1994), CO relaxes vascular smooth muscle by activating soluble guanylyl cyclase through binding to its heme component. Hence, CO would share with NO both target and second messenger (i.e. cyclic GMP). This possibility, however, is seemingly at variance with the finding that certain blood vessels respond unevenly (see Utz & Ullrich, 1991), or not at all (Brian *et al.*, 1994), to CO, notwithstanding the fact that they relax consistently to NO. Our work, comprising several lines of investigation in the ductus arteriosus (Coceani, 1994), suggests instead that CO interferes with a cytochrome P450-linked mono-oxygenase reaction serving as a limiting step in the formation of the constrictor endothelin-1 (ET-1) (Coceani *et al.*, 1996).

In the present investigation, we have addressed this issue by measuring concomitantly changes in muscle tension and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content in response to CO, both before and during exposure to monochromatic light at 450 nm as well as through treatment with guanylyl cyclase inhibitors. Experiments were carried out in the lamb ductus arteriosus which is relaxed potently by CO (Coceani *et al.*, 1988). In addition, this vessel has both prospective targets for the agent, viz. the cytochrome P450/ET-1-based contractile mechanism (Coceani, 1994; Coceani *et al.*, 1996) and the guanylyl cyclase GMP-based relaxing mechanism (Coburn *et al.*, 1986; Walsh & Mentzer, 1987; Coceani *et al.*, 1994). Hence, the ductus appears optimally suited for studying the mechanism of action of CO.

Methods

General procedure

Experiments were performed on near-term pregnant sheep (133–139 days gestation; term 145 days) of Targhee or Dorset crossbreed. The procedures for anaesthesia, Caesarean delivery

[1]Author for correspondence.

of the foetuses, and isolation of the ductus arteriosus have been published (Coceani *et al.*, 1986). The ductus was freed of loose connective tissue, opened, and then cut perpendicularly to the main axis to yield one or two strips, depending on its length. However, only one strip was used for each of these experiments. Ductal strips were prepared intact or were trimmed down to the medial layer, taking advantage of natural cleavage planes to remove the intima and the adventitia (Coceani *et al.*, 1986). The resulting preparation, whole or trimmed, was mounted in a 5 ml organ bath between a stationary glass rod and an isometric tension transducer (Grass FT-03C) coupled to a Grass polygraph. The bath, which was made of glass, had a water jacket to keep the temperature constant at 37°C and, when required, could be lowered rapidly to freeze-clamp the tissue (see below). The initial load was applied in a single step (intact ductus, 1.5–1.6 g; 1 g weight = 9.8 mN) or a series of steps (ductal muscle alone, 0.7–1.2 g), and preparations were stretched by about 50% of the original length to obtain optimal tension output (Somlyo & Somlyo, 1964). Throughout this procedure care was taken not to damage the endothelium in preparations of the whole ductus.

Tissues were equilibrated in Krebs solution containing 2.5% O_2 plus 5% CO_2 and balance N_2, and the same gas mixture or mixtures with a higher oxygen content (30 and 95%) were employed in the actual experiment. The partial pressure of O_2 (Po_2) was measured with an Instrumentation Laboratory gas analyser (mod. 1301) and was 14–26, 180–252, and 620–720 mmHg (pH 7.4 in all cases) when using, respectively, gas mixtures with 2.5, 30, and 95% O_2. Both the fluid reservoir and organ bath were continuously bubbled with the required gas mixture, and the perfusion rate through the bath was approximately 2 ml min^{-1}. In those instances in which carbon monoxide was tested on the ductus, the flow of fluid was stopped and a CO-O_2 mixture (7.8–8% CO/32.8–33.6% O_2/8.9–9% CO_2 in N_2) was delivered to the bath. This mixture was bubbled alone since it gave values of Po_2 and pH close to those obtained with the reference mixture of 30% O_2–5% CO_2 in N_2. The calculated final concentration of CO was 65 μM; however, this value may be slightly overestimated due to the reaction of CO with oxygen in the Krebs medium. Precautions were taken to avoid any leakage of carbon monoxide into the ambient air (Coceani *et al.*, 1984). The room was kept dark-ened throughout the experiments.

All tests were carried out comparatively in intact strips and smooth muscle strips. The latter preparation has both haemoproteins (i.e. cytochrome P450 and guanylyl cyclase) (Coceani *et al.*, 1988, 1994) functioning as a potential target for CO. Hence, it enables the study of CO action without any interference from agents formed outside the muscle layer, specifically in the endothelium.

Photoactivation

Ductal strips were exposed to monochromatic light at 450 nm, both in the absence and presence of CO. This wavelength was chosen since it ensures maximal reversal of the CO relaxation (Coceani *et al.*, 1988). Illumination was provided by a 75-W xenon-arc lamp (Osram, mod. XBO 75 W/2) and, to obtain even exposure of the tissue, the far side of the organ bath was lined with aluminum foil. The lamp was fitted inside a water-cooled housing and was energized with a 150-W regulated power supply. Before reaching the tissue, the light beam was first passed through a monochromator and was then focused over an appropriately narrow band. The light output from the monochromator was measured with a laser power monitor (Ophir, mod. 300 A/W) and its intensity was set at 52 mW.

During experiments, illumination was continued until reversal of the carbon monoxide relaxation had reached a maximum and the response had stabilized. In certain cases, illumination was also performed in the absence of carbon monoxide to determine any direct effect on the tissue. The temperature of the fluid inside the bath was measured in control tests and did not change through exposure to light.

Solutions and drugs

The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1, $MgSO_4$ 0.9, dextrose 11.1 and $NaHCO_3$ 25. The pH of the solution was 7.4 after equilibration with gas mixtures containing 5% CO_2.

The following inhibitors were used: indomethacin (Sigma); methylene blue (Sigma); 6-anilino-5,8-quinolinedione (LY-83583; Calbiochem); N^G-monomethyl-L-arginine acetate (L-NMMA; courtesy of Dr S. Moncada, Wellcome Research Lab.); and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma). Indomethacin is a cyclo-oxygenase inhibitor, while the remaining agents interfere with the NO/cyclic GMP system by inhibiting the synthesis (L-NMMA; L-NAME) or action (methylene blue; LY-83583) of NO. Their concentration was selected from previous studies (Mülsch *et al.*, 1988; Coceani *et al.*, 1994). Sodium nitroprusside (SNP; Sigma) was used as a NO donor.

Indomethacin and LY-83583 were dissolved in distilled ethanol (10 and 5 mg ml^{-1}, respectively) prior to preparation of the final solution in Krebs medium. Other compounds dissolved readily in saline or Krebs medium, and solutions were prepared as required on the day of the experiment. In the case of methylene blue, the solution was prepared in a darkened room to limit the formation of reactive oxygen species (Marshall *et al.*, 1988). Likewise, solutions of LY-83583 and sodium nitroprusside (SNP) were protected from light.

Doses of all compounds are given in molar concentrations and refer to their final concentration in the bath.

Measurement of cyclic GMP content

Cyclic GMP was measured with a radioimmunoassay kit (^{125}I-labelled ligand; DuPont) in ductal strips that had been set up in the special drop-away bath and freeze-clamped at an appropriate time during the recording. Brass plates precooled in liquid nitrogen were used for this rapid freezing. The frozen tissue was ground to a powder, dispersed in 6% trichloroacetic acid, and sonicated (Brenson, mod. W-140; 120 s at 20 KHz) at 4°C. The homogenate was left on ice for 1 h and then centrifuged (2500 *g* for 15 min). The resulting supernatant was extracted 5 times with 2 vol of water-saturated diethyl ether and, after discarding the ether phase, the water phase was lyophilized. The dry residue was reconstituted in 0.2 ml of sodium acetate buffer (50 mM; pH 6.2) and aliquots of this solution were treated with a mixture of acetic anhydride and triethylamine (1:2 v/v) before the assay. Cyclic GMP standard was also acetylated for preparation of a reference curve. Extraction and radioimmunoassay procedures had been validated previously (Coceani *et al.*, 1996). Furthermore, it was ascertained that none of the compounds tested on the ductus interferes with the assay. Assay values are given without any correction since there is no loss of the nucleotide through the work-up of samples (Coceani *et al.*, 1996).

Analysis of data

Contractile tension, which varied depending on the preparation and the experimental condition (see Results), is given after correction for the applied tension. Values are expressed as the mean ± s.e.mean. Statistical comparison of two means was made with Student's *t* test for paired or unpaired observations. Multiple comparisons were made with an analysis of variance (ANOVA) and Duncan's multiple range test. Differences are considered significant for $P<0.05$.

Results

Muscle tension and cyclic GMP content: effect of oxygen and indomethacin

As expected from data in the literature (Clyman, 1980; Coceani *et al.*, 1986), the intact ductus contracted upon raising the

oxygen concentration of the medium from 2.5 to 95% O_2 (Figure 1a), and the resulting tension did not differ significantly from that occurring with excess potassium (55 mM) (see Coceani *et al.*, 1986). A comparable contraction was seen with tissues exposed to 30% O_2 in the presence of indomethacin (2.8 μM) (Figure 2a). The cyclic GMP content, however, showed no obvious variation with any of these treatments and, specifically, was not affected by changes in the oxygen tension (Figure 1b and 2b). Preparations consisting of muscle only behaved as the intact preparations in contracting to 30% O_2 plus indomethacin (see Coceani *et al.*, 1986). However, due to their uneven thickness, tension output was more variable among experiments (Figure 3a). In addition, the cyclic GMP content was generally lower (difference close to significance with a t value of 2.20 when a value of 2.36 is required for $P<0.05$) with muscle bundles compared to the whole vessel (compare Figure 3b with Figure 2b). With either preparation and under all test conditions, contractile tension persisted unabated throughout the period of observation (max. 5 h). Nevertheless, in the actual study, tissues were kept in medium gassed with 30% O_2 and were also treated with indomethacin to eliminate any interference from the relaxant prostaglandin E_2 (PGE_2; see Coceani, 1994).

Effect of methylene blue and LY-83583

Methylene blue and LY-83583 (both at 10 μM) increased little, or not at all, the contractile tone of the oxygen/indomethacin-treated ductus, regardless of whether the vessel was intact (Figure 2a) or trimmed down to the muscle (Figure 3a). However, the two compounds caused a reduction in the basal content of cyclic GMP, though the actual change did not reach significance (Figures 2b and 3b).

Effect of L-NMMA and L-NAME

Previous work has shown that NO is generated spontaneously within the ductus and exerts a tonic relaxant effect (Coceani *et al.*, 1994). To remove this stimulation on guanylyl cyclase and a potential complication in the analysis of the CO response, some intact preparations were studied after adding L-NMMA or L-NAME (at 100 μM) to the medium. As evident from Figure 4a, both agents did not alter, at least to a major degree, the concentration resulting from the combined effect of oxygen and indomethacin (compare with Figure 2). However, they reduced the formation of cyclic GMP, and the resultant levels (Figure 4b) were lower ($P<0.05$) than those measured during treatment with either methylene blue or LY-83583 (see Figure 2).

Effects of light, guanylyl cyclase inhibitors, and NO synthesis inhibitors on the response to CO

In agreement with previous findings (Coceani *et al.*, 1984; 1988), CO reversed, completely or nearly completely, the tension developed by both the intact ductus (Figure 5a) and muscle bundles (Figure 6a). With either preparation, the loss in

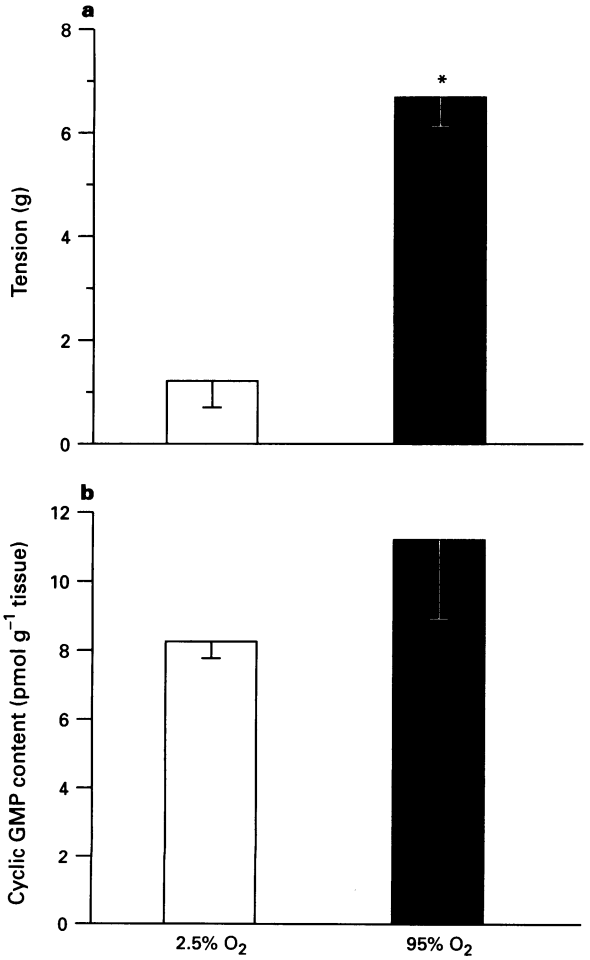

Figure 1 Effect of oxygen on contractile tension (a) and cyclic GMP content (b) in intact strips of the lamb ductus arteriosus. Strips were freeze-clamped while recording mechanical tension in an organ bath (see Methods), and different tissues were used for the two conditions ($n=3$ for each group). A significant difference between groups is indicated with an asterisk.

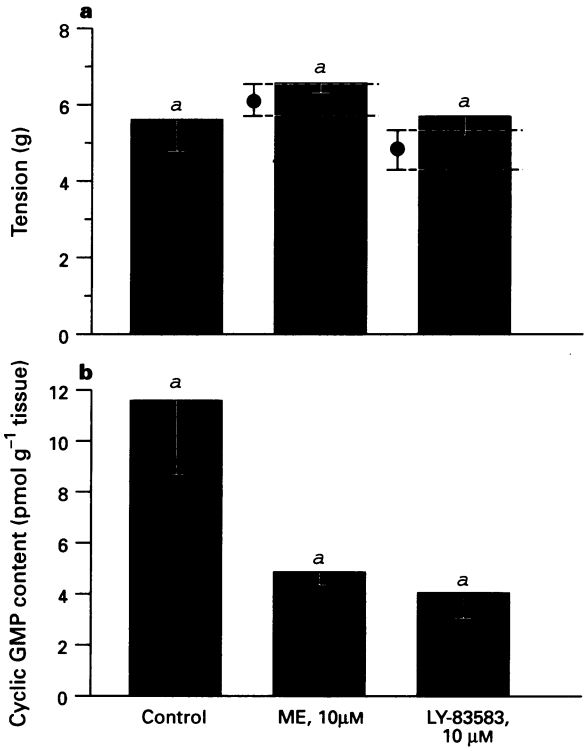

Figure 2 Effect of methylene blue (ME) and LY-83583 on contractile tension (a) and cyclic GMP content (b) in intact strips of lamb ductus arteriosus. Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). Strips were freeze-clamped while recording mechanical tension in an organ bath (see Methods), and different tissues were used for control ($n=4$) and treatment ($n=3$ for each group). Horizontal interrupted lines indicate contractile tension before treatment with either ME or LY-83583 (in both cases, difference is not significant). Note that the control group does not include one experiment in which cyclic GMP content (29.04 pmol g^{-1} tissue) departed greatly from the norm. This experiment had nothing unusual, except for the fact that the foetus belonged to a quadruplet pregnancy with individual animals of exceptional size (body weight between 3 and 4 kg). In either panel, difference among groups is not significant (same letter is shown above all columns).

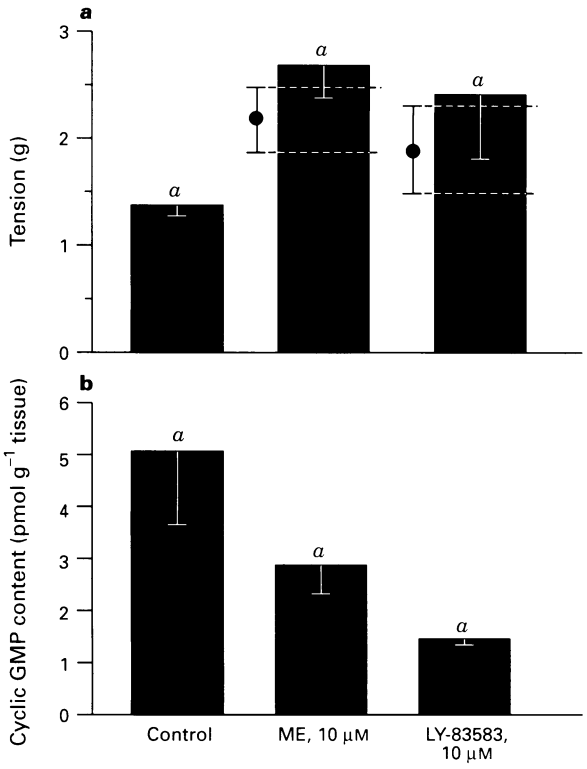

Figure 3 Effect of methylene blue (ME) and LY-83583 on contractile tension (a) and cyclic GMP content (b) in muscle strips of the lamb ductus arteriosus. Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). Strips were freeze-clamped while recording mechanical tension in an organ bath (see Methods), and different tissues were used for control ($n=5$) and treatment ($n=4$ for each group). Horizontal interrupted lines indicate contractile tension before treatment with either ME ($P<0.05$ after treatment) or LY-83583 (difference not significant after treatment). In either panel, different letters would indicate a significant difference.

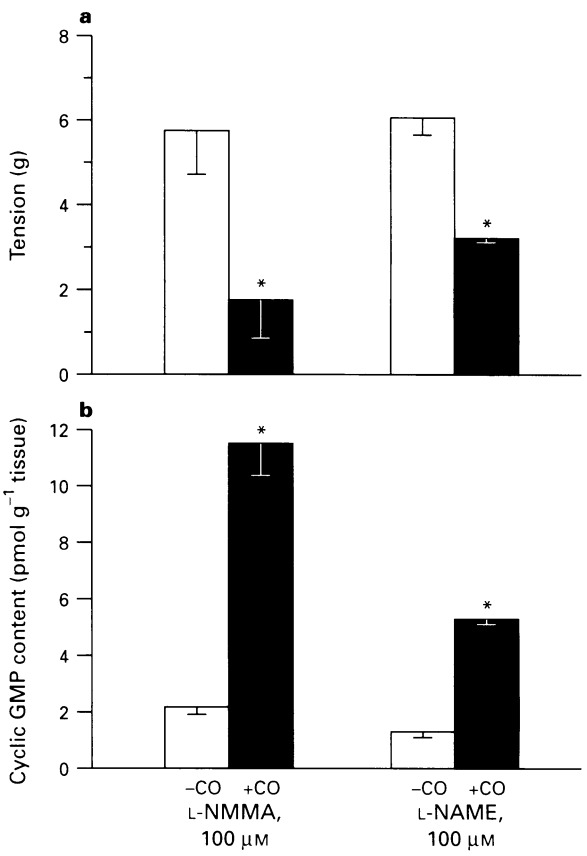

Figure 4 Effect of L-NMMA and L-NAME on the response of intact strips of the lamb ductus arteriosus to CO (65 μM). Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). (a) Contractile tension before (open column) and during (solid column) CO treatment; (b) cyclic GMP content before (open column) and during (solid column) CO treatment ($n=3$ for each group in both panels). Changes in muscle tension in response to CO were recorded from the same tissue, while different tissues were used for the measurement of cyclic GMP. A significant difference due to CO in each group is indicated with an asterisk. Note that CO-induced reversal of muscle tension was $75\pm10\%$ and $44\pm2\%$ in the presence of, respectively, L-NMMA and L-NAME, and that only the latter response was significantly different ($P<0.01$) from control (tension reversal, $88\pm2\%$ when combining all experiments of Figure 5a).

tone started after short delay (mean, 1.9; range, 1–3.5 min), progressed rapidly (mean, 9; range 4–19 min) to a peak, and was persistent. Concomitant with the relaxation there was a trend for the cyclic GMP content to rise, but the elevation remained insignificant (Figure 5b) or, at best, approached significance (Figure 6b; t value was 2.07 when a value of 2.20 is required for $P<0.05$). Unlike CO, SNP (i.e. NO) caused a marked accumulation of cyclic GMP and levels correlated positively with the degree of relaxation (Figure 7). Illumination of the CO-relaxed ductus, whole or trimmed, with monochromatic light at 450 nm restored in large part (79 ± 4 and $75\pm5\%$ for intact and smooth muscle strips, respectively) and fairly rapidly (mean, 15; range, 9–24 min) the original tone (Figure 5a and 6a) and, at the same time, abolished any upward trend in cyclic GMP levels (Figure 5b and 6b). Light by itself, on the other hand, did not modify either the tone or the cyclic GMP content of ductal muscle (Figures 6a,b), thus confirming the specifity of the response in the presence of CO.

Methylene blue reduced only slightly the magnitude of the CO-induced relaxation in both intact (Figure 8a) and smooth muscle (Figure 9a) strips. Accordingly, there was a marginal increase in latency (mean, 2.5; range, 1–6 min) and time to peak (mean, 11; range, 4–23 min) of the response. Treated tissues were still able to generate a greater amount of cyclic GMP upon exposure to CO (Figures 8b and 9b). However, the levels attained were variable and straddled the range seen with the untreated tissues under basal conditions (see Figures 2b and 3b). In addition, cyclic GMP accumulation and CO relaxation were not related (Figure 9b). LY-83583 was generally more effective than methylene blue in inhibiting the CO relaxation and, in two experiments with muscle strips, the inhibition was nearly complete (Figures 8 and 10). The response of LY-83583-treated tissues also appeared slower in onset (mean, 3.2; range 1.5–5 min) and development (mean, 12; range, 6–19 min). The pattern of cyclic GMP changes paralleled that seen with methylene blue, and accordingly, the nucleotide content showed no relationship with the magnitude of the relaxation (Figure 10b).

CO relaxation developed virtually unabated in ductal strips pretreated with L-NMMA (Figure 4a). Coincidentally, there was a 5 fold increase in cyclic GMP (Figure 4b) but, in spite of this, levels did not exceed the range seen with control tissues (see Figure 2b). Conversely, L-NAME reduced the CO relaxation, though it still allowed the cyclic nucleotide content to rise (Figure 4). Whether curtailed or not, the CO relaxation retained a normal time course through treatment with the NO synthesis inhibitors.

Discussion

This study confirms the existence in the ductus arteriosus of a functional guanylyl cyclase which is amenable to activation by NO and possibly other agents. Consistent with our conclusion is the finding that tissue levels of cyclic GMP decrease upon treatment with compounds interfering with the synthesis (L-

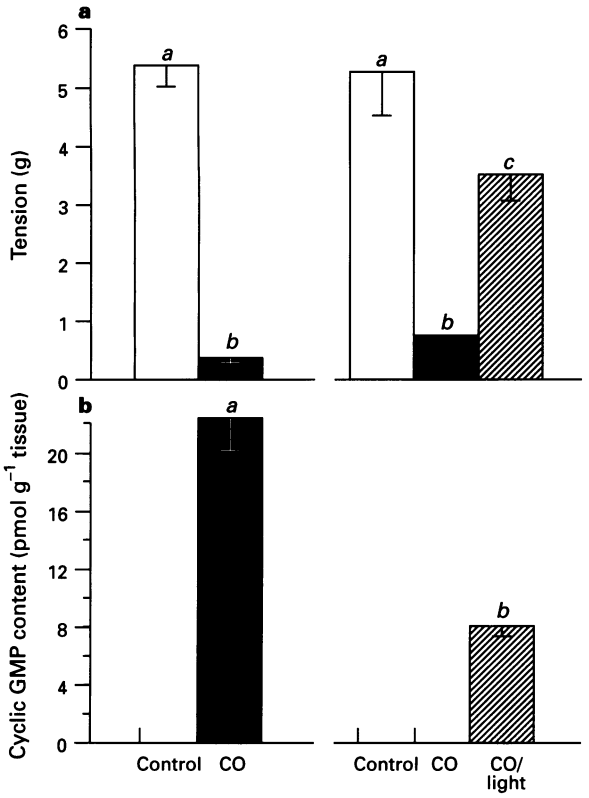

Figure 5 Intact strip of lamb ductus arteriosus. Effect of CO (65 μM) on contractile tension (a) and cyclic GMP content (b) before and during exposure to monochromatic light at 450 nm. Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). Control (open column); CO treatment (solid column); illumination during CO treatment (hatched column). Different strips were used in the two series (control/CO, $n=7$; control/CO/CO plus light, $n=5$), and cyclic GMP levels (measured in tissues freeze-clamped while recording mechanical tension, see Methods) apply to the last condition in either series. A significant difference within each series (tension values) or among series (cyclic GMP values) is indicated with different letters.

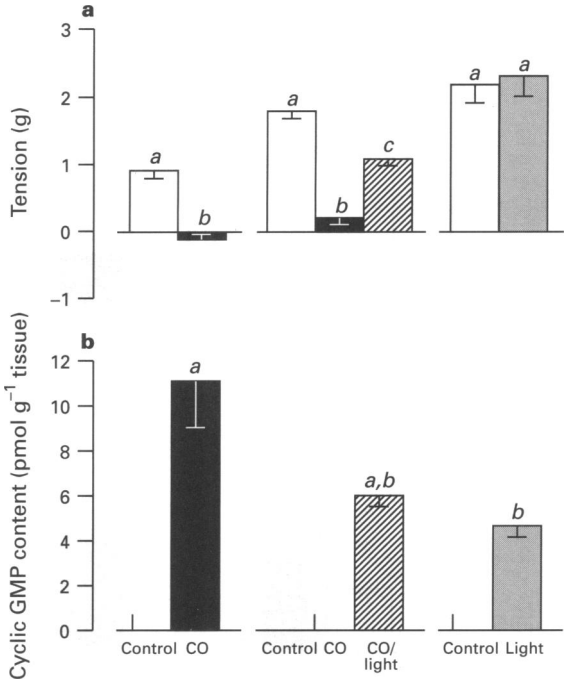

Figure 6 Effect of CO (65 μM), with or without concomitant exposure to monochromatic light at 450 nm, and light alone (at 450 nm) on contractile tension (a) and cyclic GMP content (b) in muscle strips of the lamb ductus arteriosus. Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). Control (open column); CO treatment (solid column); illumination during CO treatment (hatched column); illumination alone (stippled column). Different strips were used in the three series (control/CO, $n=8$; control/CO/CO plus light, $n=7$; light alone, $n=3$), and cyclic GMP levels (measured in tissues freeze-clamped while recording mechanical tension, see Methods) refer to the last condition in each series. A significant difference within each series (tension values) or among series (cyclic GMP values) is indicated by different letters.

NMMA, L-NAME) or action (methylene blue, LY-83583) of NO. In addition, as expected from the arrangement of the NO system in blood vessels including the ductus arteriosus (Coceani *et al.*, 1994), the yield of the nucleotide is lower in preparations of only muscle than in those of the entire vessel. Hence, the idea of the endothelium being a prime source of NO in the ductus is validated (Coceani *et al.*, 1994). Our results also support the view that CO may activate ductal guanylyl cyclase. Compared to NO, however, this activation varies among experiments and, in general, it is much weaker. The original question of whether guanylyl cyclase mediates the CO-induced relaxation cannot be answered in an unequivocal manner, because methylene blue and LY-83583 had an inhibitory effect, albeit inconsistent and often marginal. On the other hand, illumination of the ductus reversed nearly completely the changes in muscle tension and cyclic GMP content due to CO, and this effect occurred with a wavelength (i.e. 450 nm) which was not optimal for the dissociation of the guanylyl/CO complex (i.e. 422 nm). This notwithstanding, it is significant that tissues treated with methylene blue and LY-83583 retained, whether in part or in full, the capability to relax to CO and that any elevation in cyclic GMP occurring in the course of such relaxation did not exceed the range of values seen with control tissues. Furthermore, the nucleotide content was not related to the magnitude of the relaxation. Together, the latter data argue against a role, or at least a prime role, of guanylyl cyclase as the target for CO. In addition, considering that the cyclic GMP content is not altered by changes in

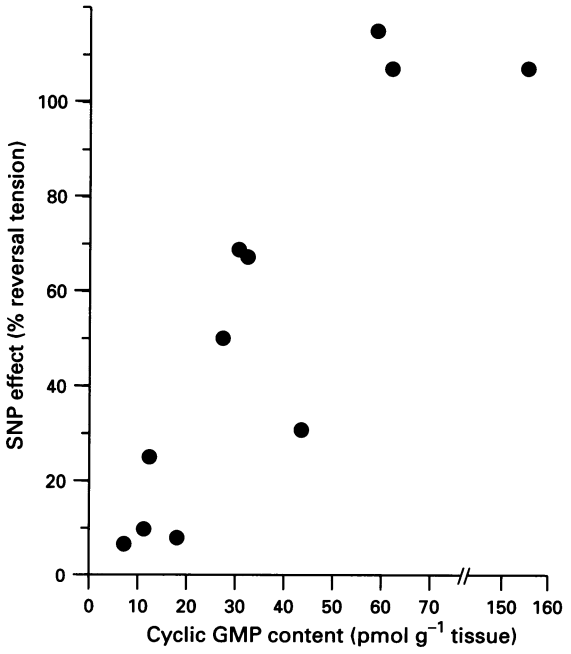

Figure 7 Intact strip of lamb ductus arteriosus. Relationship between reversal of contractile tone and cyclic GMP accumulation during treatment with sodium nitroprusside (SNP, concentration range, 0.003–1 μM). Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). Note that each point refers to a different experiment and that correlation coefficient is 0.72 ($P=0.01$).

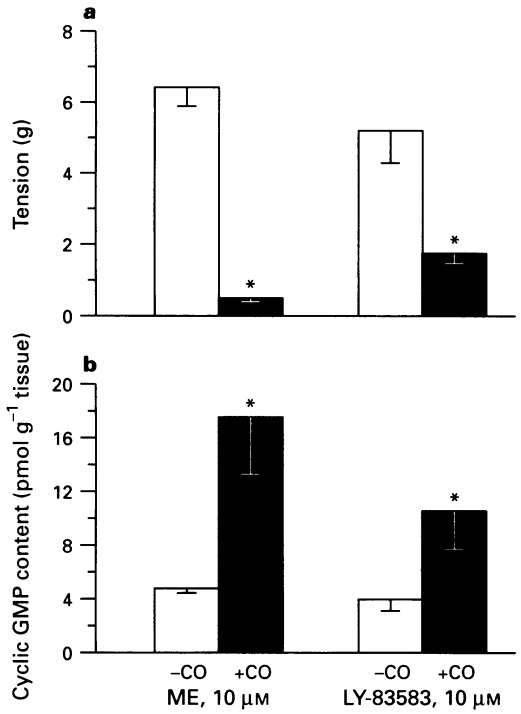

Figure 8 Effect of methylene blue (ME) and LY-83583 on the response of intact strips of the lamb ductus arteriosus to CO (65 μM). Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). (a) Contractile tension before (open columns) and during (solid columns) CO treatment; (b) cyclic GMP content before (open columns) and during (solid columns) CO treatment ($n=3$ for each group in both panels). Changes in muscle tension in response to CO were recorded from the same tissue, while different tissues were used for the measurement of cyclic GMP (reference value before CO is the same as Figure 2). A significant difference due to CO in each group is indicated with an asterisk. Note that CO-induced reversal of tension was 104±1% before adding ME (compared to 91±2% with ME; $P<0.05$) and 98±1% before adding LY-83583 (compared to 65±10% with LY-83583; difference is not significant) to the medium.

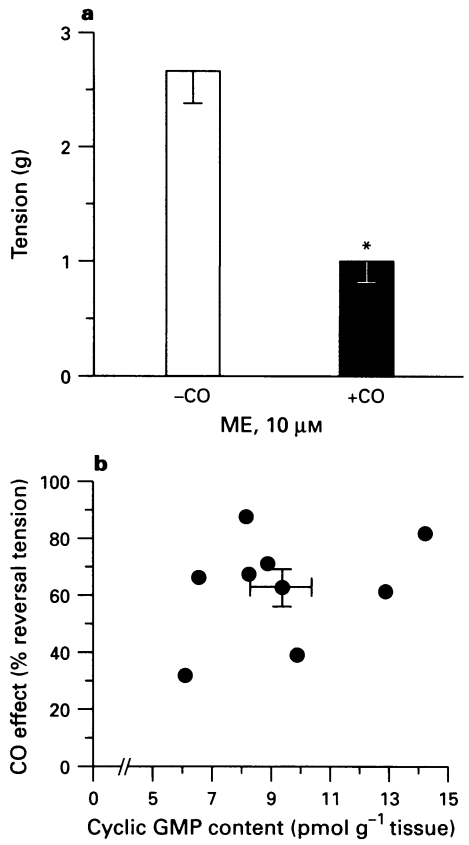

Figure 9 Effect of methylene blue (ME) on the response of muscle strips of the lamb ductus arteriosus to CO (65 μM). Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). (a) Contractile tension before (open column) and during (solid column) CO treatment; (b) relationship between cyclic GMP content and CO-induced relaxation (individual values shown together with mean± s.e.mean). Data in the two panels apply to the same tissue ($n=8$). In (a), a significant difference is indicated with an asterisk. CO-induced reversal of tension was 83±7% before adding ME to the medium and became 62±6% in the presence of ME ($P<0.01$). In (b), the correlation coefficient is 0.31 (P not significant). Note that as a group cyclic GMP values are significantly higher ($P<0.01$) than those measured in ME-treated tissues prior to CO (see Figure 3b).

oxygen tension (these results) and knowing at the same time that oxygen does not bind to guanylyl cyclase (Stone & Marletta, 1994), it is reasonable to assume that the opposing actions of oxygen and CO on ductal tone are exerted on an alternative site. According to our data, the cytochrome P450-based mono-oxygenase reaction controlling ET-1 synthesis (Coceani, 1994; Coceani *et al.*, 1996) is such a site. From this premise, our discussion will concern the following three issues: the reason for the uneven effectiveness of methylene blue versus LY-83583 in inhibiting guanylyl cyclase activity, specifically the CO-stimulated activity; the mechanism by which CO relaxes the ductus arteriosus; and the implications of our findings for the regulation of ductal tone.

Methylene blue and LY-83583 are utilized widely as inhibitors of the NO/cyclic GMP system, despite uncertainties on their mode of action and the attendant possible difficulties in the interpretation of experimental data. Both compounds generate superoxide radicals on contact with tissues, and this event is thought to be critical for the inhibition (Marshall *et al.*, 1988; Mülsch *et al.*, 1988; Wolin *et al.*, 1990; Marczin 1992). However, there is no consensus on whether free radicals owe their effect to trapping of NO, inhibition of guanylyl cyclase, or both processes combined (Mülsch *et al.*, 1988; Wolin *et al.*, 1990). In fact, according to some authors, inhibition of the enzyme is a secondary, and functionally unimportant, effect, at least in the case of methylene blue (Marshall *et al.*, 1988; Marczin *et al.*, 1992). Another complication is that any direct action on guanylyl cyclase does not involve the heme-containing subunit, hence inhibitors and NO cannot compete for the same site (Brüne *et al.*, 1990). Furthermore, whatever the mechanism, the actual susceptibility of tissues to these inhibitors is probably conditioned by the efficiency of local enzyme systems responsible for the de-activation of free radicals (Marshall *et al.*, 1988; Wolin *et al.*, 1990; Marczin *et al.*, 1992). Accordingly, inhibitors are variably effective against NO depending on the vessel preparation, the source of the agent, and the magnitude of the activation (Gruetter *et al.*, 1981; Marshall *et al.*, 1988). When considering all these facts, one may explain not only inconsistencies in the literature, but also our findings with methylene blue and LY-83583 in the ductus. Conceivably, accumulation of cyclic GMP at rest reflects a steady activation from endogenous NO (see Coceani *et al.*, 1994). Therefore, by interfering with NO action in two possible ways, the inhibitors can effectively disrupt this process. Conversely, when CO binds to the haemoprotein subunit of guanylyl cyclase, the only factor to condition the subsequent response is the alteration in enzyme function due to the direct effect of the inhibitors. In our preparation, the impact of this factor would appear greater with LY-83583 than methylene blue.

The present results are consistent with a role of a cytochrome P450-based mono-oxygenase reaction in the contraction of the ductus arteriosus to oxygen. By extension, the same reaction is implicated in the relaxation of the vessel to CO. The idea of guanylyl cyclase being the exclusive target for CO, as

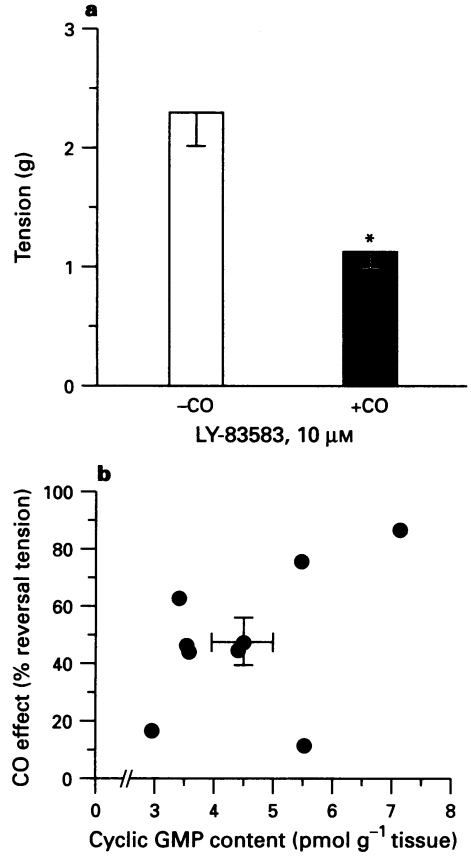

Figure 10 Effect of LY-83583 on the response of muscle strips of the lamb ductus arteriosus to CO (65 μM). Krebs medium was gassed with 30% O_2 and contained indomethacin (2.8 μM). (a) Contractile tension before (open column) and during (solid column) CO treatment; (b) relationship between cyclic GMP content and CO-induced relaxation (individual values are shown together with mean ± s.e.mean). Data in the two panels apply to the same tissues ($n=8$). In (a), a significant difference is indicated with an asterisk. CO-induced reversal of tension was 92 ± 9% before adding LY-83583 to the medium and became 46 ± 8% in the presence of LY-83583 ($P<0.05$). In (b), the correlation coefficient is 0.48 (P not significant). Note that as a group cyclic GMP values are significantly higher ($P<0.01$) than those measured in LY-83583-treated tissues prior to CO (see Figure 3b).

proposed by some investigators (Brüne *et al.*, 1990; Gräser *et al.*, 1990; Furchgott & Jothianandan, 1991; Ewing *et al.*, 1994), does not appear tenable, at least in the case of the ductus. In support of our position are the insignificant accumulation of cyclic GMP in the CO-treated tissue, the lack of any correlation between nucleotide content and magnitude of the CO relaxation, and the general ineffectiveness of methylene blue and LY-83538 in curtailing this relaxation. A single apparent incongruity is that light at 450 nm reverses the CO relaxation and any concomitant increase in cyclic GMP. It must be pointed out, however, that this wavelength, though most effective in cleaving off CO from a P450 haemoprotein, may still interfere with the guanylyl cyclase/CO complex and any associated ligand-induced stimulation. The latter action may be particularly evident when dealing with a modest stimulation. While arguing against the involvement of guanylyl cyclase in the CO-induced relaxation of the ductus, our study leaves open the possibility of an accessory role for this mechanism or even a major role under certain conditions. One such condition could be extreme hypoxia when, according to our previous work (Coceani *et al.*, 1996), the cytochrome P450/ET-1 mechanism is little active and contractile tone is sustained primarily by the withdrawal of relaxing influences (e.g. PGE_2, NO). Significant in this connection is the finding that the CO-induced relaxation of the hypoxic ductus, unlike that of the normally oxygenated ductus, is reversed marginally, or not at al, by either polychromatic (Coceani *et al.*, 1984) or monochromatic (at 450 nm; F. Coceani, unpublished observations) light. Conceivably, photodissociation has a greater impact when muscle tone is sustained by the presence of a constrictor rather than the absence of a relaxant.

Besides defining potential targets for CO, our study has general implications for the regulation of vascular tone. Probably, the cytochrome P450/ET-1-based contractile mechanism, demonstrated by us in the ductus arteriosus (Coceani *et al.*, 1994; 1996), is not limited to this site. For example, one may surmise its presence in vessels, whether in the foetus or the adult, which like the ductus are constricted by oxygen. Furthermore, as evidenced by work in the foetal ductus venosus (Adeagbo *et al.*, 1990), this mechanism may not be linked exclusively to oxygen. It could exist widely in a latent state and could become functional under certain physiologic or pathophysiologic conditions, specifically conditions in which P450 haemoproteins are induced. These possibilities would warrant experimental verification.

A final point deserving a comment relates to the inhibitory effect of L-NAME, but not L-NMMA, on the relaxant response of the ductus to CO. No explanation can be given for this unexpected observation. However, it may not be fortuitous that L-NAME differs from L-NMMA in that it inhibits NO synthesis by interfering with an NADPH-dependent reduction of molecular oxygen (Mayer, 1994), hence an event common to all mono-oxygenase reactions. Perhaps, the lesser effectiveness of CO in the presence of L-NAME is due to the lack of a fully functional target.

In conclusion, the present study strengthens the concept of a cytochrome P450-based mono-oxygenase reaction playing a key role in the contraction of the ductus arteriosus to oxygen. The same reaction also serves as the main target for CO. Hence, the idea of guanylyl cyclase/cyclic GMP system being the exclusive effector for this agent is not confirmed. The actual significance of this contractile mechanism to general vasoregulation in the foetus and the adult remains to be clarified.

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