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Cyclic GMP-independent relaxation and hyperpolarization with acetylcholine in guinea-pig coronary artery

D.M. Eckman, *J.S. Weinert, *I.L.O. Buxton & [1]K.D. Keef

Department of Physiology, University of Nevada, Reno Reno, NV 89557 and *Department of Pharmacology, University of Nevada, Reno Reno, NV 89557, U.S.A.

1 The effects of acetylcholine (ACh) on membrane potential, relaxation and cyclic GMP levels were compared to the NO donor L-nitrosocysteine (Cys-NO) in segments of guinea-pig coronary artery.

2 ACh and Cys-NO produced concentration-dependent relaxations of muscles contracted with the H_1 receptor agonist, 2-(2-aminoethyl)pyridine (AEP, 0.35 mM). The relaxation to ACh was unchanged in the presence of N^G-monomethyl-L-arginine (L-NMMA; 350 μM) or indomethacin (3 μM).

3 Oxyhaemoglobin (HbO; 20 μM) alone or in combination with L-NMMA increased the EC_{50} for ACh-induced relaxation whereas relaxation with Cys-NO was almost completely abolished with HbO.

4 Scorpion venom (SV; 8.7 μg ml^{-1}) increased the EC_{50} for relaxation with ACh but not Cys-NO. Combined L-NMMA, HbO and SV produced nearly complete abolition of ACh-induced relaxations.

5 Basal cyclic GMP levels (i.e., 20 pmol mg^{-1} protein) were significantly increased following addition of either ACh (190 pmol mg^{-1} protein) or Cys-NO (240 pmol mg^{-1} protein). L-NMMA significantly reduced the rise of cyclic GMP with ACh but not Cys-NO. In contrast, SV did not significantly reduce the rise in cyclic GMP produced with ACh. In the combined presence of L-NMMA and HbO neither ACh nor Cys-NO produced a significant increase in cyclic GMP levels.

6 ACh gave rise to significantly greater membrane hyperpolarization than Cys-NO both in the presence and absence of AEP. Combined L-NMMA and HbO did not reduce the amplitude of hyperpolarization with ACh.

7 These data indicate that some but not all of the actions of ACh in the coronary artery can be mimicked by the NO donor, Cys-NO, suggesting that ACh releases NO as well as a second hyperpolarizing factor (i.e., EDHF). Release of NO results in a large increase in tissue cyclic GMP levels and minimal change in membrane potential whereas release of EDHF results in a large membrane hyperpolarization which is independent of changes in tissue cyclic GMP levels. Both of these pathways appear to contribute to relaxation throughout the entire ACh concentration-relaxation relationship.

Keywords: EDHF; EDRF; relaxation; membrane potential; cyclic GMP; guinea-pig coronary artery; blood vessel; endothelium

Introduction

Acetylcholine (ACh) produces relaxation in blood vessels via an endothelium-dependent mechanism (Furchgott & Zawadzki, 1980; for review see Furchgott & Vanhoutte, 1989). An important factor involved in this response is nitric oxide (NO) which is released from the endothelium following the binding of ACh to muscarinic receptors (Hynes *et al.*, 1986; Dauphin & Hamel, 1990). NO diffuses to the adjacent vascular smooth muscle cells where it stimulates soluble guanylyl cyclase activity leading to increased cyclic GMP levels (Ignarro, 1989; Moncada *et al.*, 1991). Both the relaxation and enhanced guanylyl cyclase activity produced by ACh can be mimicked by addition of authentic NO (Gruetter *et al.*, 1981; Ignarro *et al.*, 1987).

ACh has also been reported to hyperpolarize smooth muscle cells in a number of different blood vessels via an endothelium-dependent mechanism (e.g., Komori & Suzuki, 1987; Taylor & Weston, 1988; Chen *et al.*, 1988; Keef & Bowen, 1989). However, in several blood vessels the ACh-induced hyperpolarization is not mimicked by NO (Bény & Brunet, 1988; Komori *et al.*, 1988; Brayden, 1990; Brayden *et al.*, 1991; Garland & McPherson, 1992) nor can hyperpolarization be blocked by manoeuvres which antagonize the NO pathway such as oxyhaemoglobin (Komori *et al.*, 1988; Chen *et al.*, 1988; Huang *et al.*, 1988; Nishiye *et al.*, 1989) arginine analogues (Nagao & Vanhoutte, 1991; Chen *et al.*, 1991; Garland & McPherson, 1992), and methylene blue (Komori *et al.*, 1988; Chen *et al.*, 1988; Brayden, 1990; Garland & McPherson, 1992). These observations have led to the proposal that a second factor, distinct from NO, is also released from the endothelium in response to ACh stimulation. This factor has been termed 'endothelium derived hyperpolarizing factor' or EDHF (Bény & Brunet, 1988; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; Taylor & Weston, 1988). Evidence has been presented to suggest that the hyperpolarization is due to the opening of K^+ channels in the smooth muscle membrane (Kitamura & Kuriyama, 1979; Brayden, 1990; Chen & Suzuki, 1990; Chen *et al.*, 1991; Eckman *et al.*, 1992; Garland & McPherson, 1992).

There is still considerable controversy regarding the contribution of EDHF to endothelium-dependent relaxation. In some blood vessels ACh-induced relaxation is almost completely abolished in the presence of antagonists of the NO pathway such as arginine analogues, oxyhaemoglobin and methylene blue (Martin *et al.*, 1985; Chen *et al.*, 1988; Huang *et al.*, 1988; Flavahan *et al.*, 1989; Rees *et al.*, 1990). In other studies the physiological relevance of EDHF is unclear since hyperpolarization has been reported to be partially or completely blocked without reducing the relaxation response to ACh (Komori & Suzuki, 1987; Feletou & Vanhoutte, 1988; Chen *et al.*, 1988; Komori & Vanhoutte, 1990; Nagao & Vanhoutte, 1991). Finally, in some studies it has been suggested that endothelium-dependent relaxation depends upon both NO and EDHF since partial inhibition of relaxation was obtained when either the NO or EDHF pathway was blocked (Brayden, 1990; Nagao & Vanhoutte, 1991; Suzuki *et al.*, 1992).

ACh produces significant endothelium-dependent hyper-

[1] Author for correspondence at: Anderson Bldg., Room 115, Department of Physiology, University of Nevada, Reno Reno, NV 89557, U.S.A.

polarization of the guinea-pig coronary artery (Kitamura & Kuriyama, 1979; Keef & Bowen, 1989; Chen et al., 1991; Eckman et al., 1992) and we have previously suggested that EDHF plays an important role in eliciting relaxation in this tissue (Keef & Bowen, 1989). To distinguish further the NO and the EDHF pathway in this blood vessel the effects of ACh on membrane potential, contractile tension and cyclic GMP levels were compared with the NO donor L-nitrosocysteine (Cys-NO).

Methods

Contractile experiments

Male albino guinea-pigs (350–550 g) were killed by CO_2 inhalation followed by exsanguination. The heart was immediately removed and placed in cold (10°C) oxygenated Krebs solution. A 1–1.5 cm segment of the left circumflex coronary artery was dissected out and cleaned of all adhering fat and myocardial tissue. Ring segments (3 mm long) were mounted onto two triangular tungsten wires (89 μm diameter) and hung vertically in an isolated organ chamber. The bottom triangle was mounted on a stable hook while the top triangle was attached to a Gould strain guage. The bath was maintained at 37°C in Krebs solution of the following composition (mM): NaCl 118.5, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, KH_2PO_4 1.2, dextrose 11, $CaCl_2$ 2.5 and aerated with 95% O_2/5% CO_2.

A resting force of 0.3 g was applied to the guinea-pig coronary artery. In preliminary experiments this was found to stretch vessels to near the optimal length for tension development (i.e. L_o). Vessels were initially equilibrated for 2 h with alternating 4 min exposures every 15 min to the histamine H_1-receptor agonist, 2-(2-aminoethyl)pyridine (AEP 0.1–1 mM, Durant et al., 1975) and 100 mM KCl.

The experiments in which the effects of L-NMMA, HbO or scorpion venom were tested on contractile responses to ACh were performed in a sequential manner where each vessel segment served as its own control. On the other hand, experiments in which the effect of N^G-monomethyl-L-arginine (L-NMMA), oxyhaemoglobin (HbO) and scorpion venom were tested on responses to Cys-NO were undertaken in a parallel fashion where adjacent vessel segments from the same animal were used. This was done since it was often difficult to reverse completely the inhibition produced with high concentrations of Cys-NO. One artery segment therefore served as a control and the other was exposed to L-NMMA (350 μM) 20–30 min prior to contracting with AEP and addition of Cys-NO. To insure reproducibility further, each matched control and experimental tissue was exposed to the same stock solution of Cys-NO.

Cyclic GMP measurements

Cyclic GMP measurements were made on tissues under three different conditions including basal levels, during contraction with AEP and during relaxation with either ACh or Cys-NO (see inset to Figure 4). Artery segments were flash-frozen in liquid nitrogen while still attached to the stainless steel triangles. Cyclic GMP was assayed with commercially available reagents (Caymen Chemical Company, Ann Arbor, MI, U.S.A.). Samples were prepared for assay by homogenization in 6% TCA with glass Duall tissue grinders followed by extraction with water-saturated diethyl ether. Aqueous phases were then lyophilized to dryness and resuspended in 1.0 M potassium phosphate buffer (pH 7.4) before addition to duplicate microtiter plate wells. Cyclic GMP levels in samples and standards were detected following acetylation and competition between cyclic GMP and the acetylcholinesterase-linked cyclic GMP tracer for specific antiserum binding sites. The antiserum complex, linked to acetylcholinesterase, was used to cleave Ellman's reagent (5,5'-dithio-*bis*-(2-nitrobenzoic acid), and absorbance was measured at 412 nm. Cyclic GMP content of samples was determined from a standard curve constructed from determination of known amounts of cyclic GMP added to the plate. Levels of cyclic GMP are expressed as pmol cyclic GMP mg^{-1} protein (determined by method of Bradford, 1976). Duplicate variation in the cyclic GMP assay was less than 3%.

Intracellular measurements

For experiments measuring membrane potential, a 0.5–0.7 cm segment of left circumflex coronary artery was employed. Vessels rings were mounted onto two parallel wires and stretched to approximately 1.3–1.5 fold the resting diameter. All intracellular measurements were made through the adventitial surface with microelectrodes filled with 3 M KCl and having resistances between 70–100 MΩ. Intracellular measurements were made in the absence and presence of AEP-induced contractions. Impalements were judged on the basis of a rapid drop in potential upon entering the cell, a low noise level and minimal change in the electrode resistance and zero potential before and after impalement. Signals were viewed on a digital oscilloscope (Hitachi) and stored on tape with a Vetter PCM Recording Adapter attached to a video cassette recorder.

Statistics

Statistical significance was determined by the two-tailed paired or unpaired t test. The changes were considered significant at $P<0.05$. Data are expressed as mean ± s.e.mean, n values indicate the number of animals studied. The concentration of drug producing half maximal relaxation (i.e., EC_{50}) was calculated for each experiment by normalizing to the maximum amount of relaxation attained on that day. The software program InPlot (Graphpad software, Carlsbad, CA, U.S.A.) was used to calculate these EC_{50} values. This programme utilizes nonlinear regression according to the method of Marquardt (1963). However, the data in Figures 1–3 are plotted as a percentage of complete relaxation of the tissue so that the relative efficacy of the various drugs can be compared.

Drugs used

Acetylcholine HCl (ACh; Sigma, St. Louis, MO, U.S.A.), scorpion venom (*Leiurus quinquestriatus habraeus*, S.V; Sigma, St. Louis, MO, U.S.A.), 2-(2-aminoethyl)pyridine (AEP, Aldrich, Milwaukee, WI, U.S.A.), an indomethacin (Sigma), N^G-monomethyl-L-arginine, HOAc salt (L-NMMA, Calbiochem). L-Nitrosocysteine (Cys-NO) was made fresh daily according to Field et al. (1978). Haemolysate was made according to the method of Bowman & Gillespie (1982) and was stored at 2°C for up to 3 days. Since the active component of haemolysate is oxyhaemoglobin we have referred to haemolysate as oxyhaemoglobin (HbO) throughout the text. Three volume % of haemolysate solution was added to the bath to attain a final concentration of approximately 20 μM HbO.

Results

Contractile experiments

Comparison of the effect of N^G-monomethyl-L-arginine on acetylcholine- and L-nitrosocysteine-induced relaxations The concentration-relaxation relationships for ACh (0.0035–3.5 μM) and Cys-NO (0.0035–3.5 μM) were obtained by cumulative addition of drugs to segments of coronary artery contracted with AEP (100 μM). Both drugs were capable of producing full relaxation of the tissue and their potencies were very similar to one another (EC_{50} values -7.36 ± 0.12, $n = 10$ and -7.62 ± 0.16, $n = 8$ for ACh and

Cys-NO respectively, Figure 1). The ability of ACh and Cys-NO to relax vessels segments was also tested in the presence of the NO synthase (NOS) inhibitor, L-NMMA (350 μM, 25 min). This arginine analogue was used since it does not antagonize muscarinic receptors (Buxton *et al.*, 1993). The EC_{50} values for ACh and Cys-NO obtained in the presence of L-NMMA (i.e., -7.06 ± 0.13, $n = 10$ and -7.61 ± 0.11, $n = 9$ respectively) were not significantly different from their respective control values (Figure 1). The AEP-induced contraction was 137.5 ± 18.2% of control amplitude in the presence of L-NMMA suggesting that there is basal release of NO in this tissue.

Comparison of the effect of oxyhaemoglobin on acetylcholine- and L-nitrosocysteine-induced relaxations The concentration-relaxation relationships for ACh and Cys-NO were also measured following exposure to the NO scavenger, HbO (20 μM, 30 min). In the presence of HbO there was a small but significant shift to the right of the ACh relationship (-7.53 ± 0.06, $n = 18$ control versus -7.12 ± 0.08, $n = 18$ with HbO, Figure 2a). In contrast, relaxation with Cys-NO ($EC_{50} = -6.47 \pm 0.13$, $n = 12$) was almost completely abolished in the presence of HbO (Figure 2b) indicating that HbO effectively binds to the NO released from the NO donor Cys-NO. The AEP induced contraction was 158 ± 19% ($n = 12$) of control amplitude in the presence of HbO again suggesting that there is basal release of NO in this tissue.

The inability of either L-NMMA or HbO to diminsh substantially responses to ACh might be due to incomplete blockade of the NO pathway. To explore this possibility further, additional experiments were undertaken using combined L-NMMA (350 μM) and HbO (20 μM). The concentration relaxation relationship for ACh in the presence of L-NMMA and HbO was significantly to the right of the control relationship (-7.51 ± 0.13 control versus -7.04 ± 0.14, $n = 11$ with HbO and L-NMMA) but was not different from the responses observed with HbO alone (i.e., Figure 2a). These data suggest that ACh releases significant quantitities of NO since relaxations with ACh are reduced following blockade of the NO pathway. However, since relaxation is not abolished it also suggests that another factor, presumably EDHF, contributes to relaxations produced with each concentration of ACh.

Effect of scorpion venom on acetylcholine- and L-nitrosocysteine-induced relaxations We have previously reported that the crude scorpion venom (SV) from *Leiurus quinquestriatus hebraeus* (Eckman *et al.*, 1992) reduces the ACh-induced hyperpolarization in the guinea-pig coronary artery by 94% This venom contains a number of different substances known to block K^+ channels (Strong, 1990). In the present study we have investigated the effects of this venom on the concentration-relaxation relationships for ACh and Cys-NO. In the presence of SV (8.7 μg ml^{-1}, 30 min) the relaxation relationship for ACh was significantly shifted to the right (EC_{50}

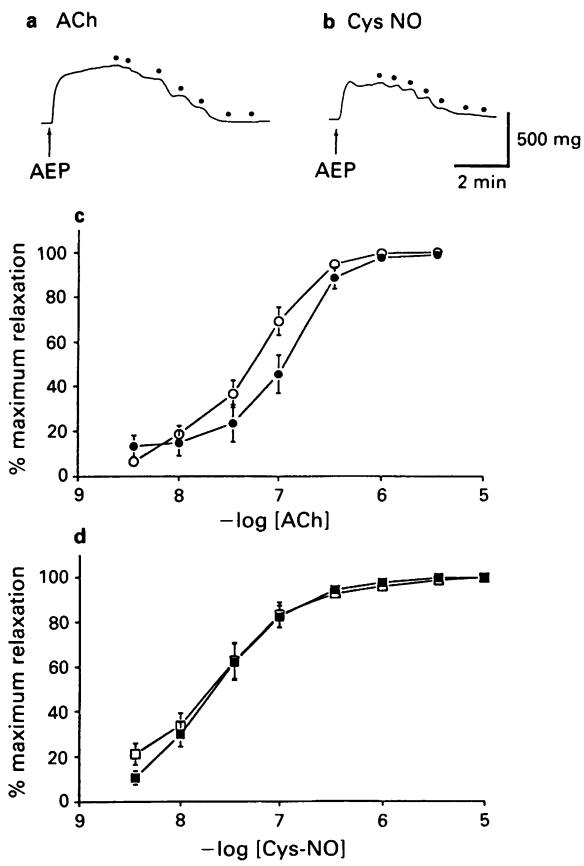

Figure 1 Comparison of the effects of N^G-monomethyl-L-arginine (L-NMMA, 350 μM) on the concentration-relaxation relationships for acetylcholine (ACh) and L-nitrosocysteine (Cys-NO). (a) and (b) Examples of the relaxation response to cumulative addition of either ACh (a) or Cys-NO (b) in two different blood vessels contracted with 2-(2-aminoethyl)pyridine (AEP). (c) Concentration-relaxation relationship for ACh in the absence (○) or presence (●) of L-NMMA. The EC_{50} values for ACh-induced relaxation were not significantly different (-7.36 ± 0.12, control versus -7.06 ± 0.13, $n = 10$, L-NMMA). (d) Concentration-relaxation relationship for Cys-NO in the absence (□) or presence (■) of L-NMMA. The EC_{50} values for Cys-NO induced relaxation (-7.62 ± 0.16, $n = 8$, control versus -7.61 ± 0.11, $n = 9$, L-NMMA) were not significantly different. Shown are mean values ± s.e.

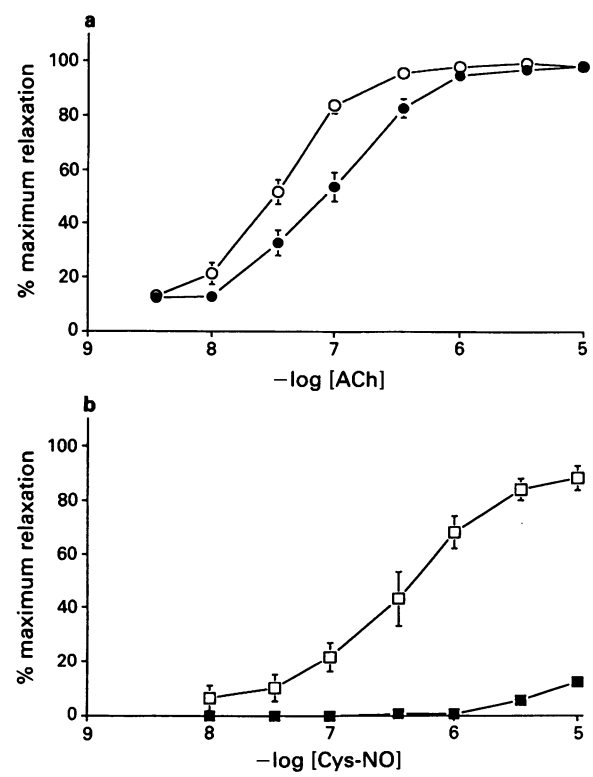

Figure 2 Comparison of the effects of oxyhaemoglobin (HbO, 20 μM) on the concentration-relaxation relationships for acetylcholine (ACh) and L-nitrosocysteine (Cys-NO). (a) Concentration-relaxation relationship for ACh in the absence (○) or presence (●) of HbO. The EC_{50} values for ACh-induced relaxation were significantly shifted to the right in the presence of HbO. (b) Concentration-relaxation relationship for Cys-NO in the absence (□) or presence (■) of HbO. The Cys-NO response was almost completely eliminated in the presence of HbO. Shown are mean values ± s.e.

-7.14 ± 0.11 control, $n = 9$, versus -6.39 ± 0.22 with SV, $n = 5$, Figure 3a) whereas responses with Cys-NO were unchanged (EC_{50} -7.49 ± 0.15 control versus -7.35 ± 0.06 with SV, $n = 4$, Figure 3b). Since SV significantly reduced responses to ACh it suggests that the EDHF pathway also contributes significantly to the ACh-induced relaxation.

Effect of combined N^G-monomethyl-L-arginine, oxyhaemoglobin and scorpion venom on acetylcholine-induced relaxation

Our results indicate that the ACh-induced relaxation persists in the presence of blockade of either the NO or the EDHF pathway. Experiments were therefore undertaken to determine the extent to which relaxation is reduced following combined blockade of both pathways. For these experiments tissues were bathed in L-NMMA (350 μM), HbO (20 μM) and SV (8.7 μg ml^{-1}) for 30 min before the concentration-relaxation relationship for ACh was measured. In the presence of all three blockers, relaxation with ACh was almost completely abolished ($n = 4$, Figure 3a). In contrast, both the control relaxation with ACh (-7.24 ± 0.08, $n = 14$) and the relaxation obtained in the presence of HbO and L-NMMA (-6.67 ± 0.15, $n = 5$) were unchanged in the presence of indomethacin (3 μM; -7.22 ± 0.25, $n = 4$ and -6.63 ± 0.11, $n = 5$, respectively).

Measurements of cyclic GMP levels

Relaxation of blood vessels by NO is known to involve stimulation of smooth muscle guanylyl cyclase activity leading to a rise in tissue cyclic GMP levels (Rapoport & Murad, 1983; Ignarro, 1989). Our results suggest that both NO and EDHF significantly contribute to relaxations induced with ACh. To assess directly the relationship of cyclic GMP to relaxations elicited with ACh and Cys-NO, experiments were undertaken in which cyclic GMP levels were measured in artery segments under the same conditions employed for contractile experiments (see methods).

Cyclic GMP levels in the tissue were measured at rest, during peak contraction with AEP and during relaxation with a concentration of ACh (0.35–1 μM) or Cys-NO (1–3.5 μM) which produced at least 90% maximum relaxation (see inset, Figure 4). Significant basal levels of cyclic GMP were detected in the absence of agonist (21 ± 5 pmol mg^{-1} protein, $n = 4$). During contraction with AEP, cyclic GMP levels were not significantly increased above the control level (33 ± 4 pmol mg^{-1} protein, $n = 4$). Upon application of ACh, cyclic GMP levels increased 5.7 fold ($n = 5$) above the level measured in the presence of AEP alone. Following application of Cys-NO, cyclic GMP levels were 7.5 fold greater ($n = 5$, Figure 4). Cyclic GMP levels in the presence of ACh and Cys-NO were not significantly different from one another.

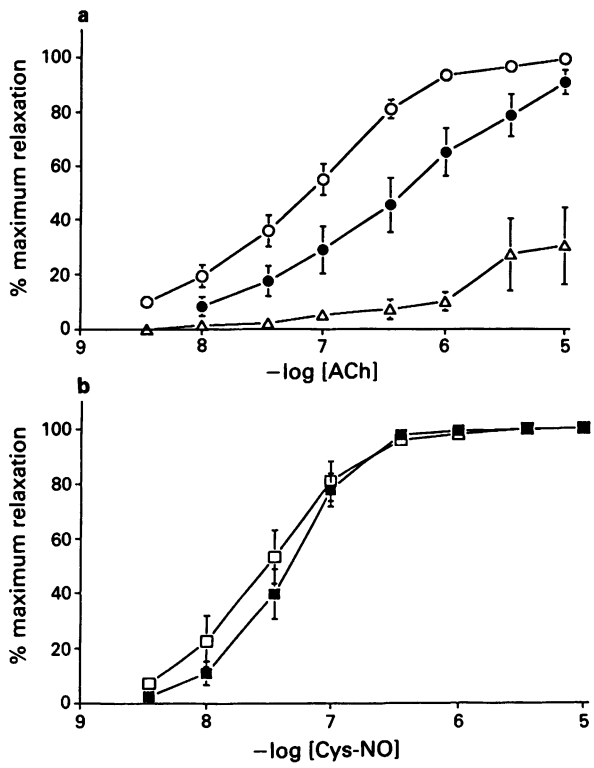

Figure 3 Comparison of the concentration-relaxation relationship for acetylcholine (ACh) and L-nitrosocysteine (Cys-NO) under various conditions. (a) ACh concentration-relaxation relationship in the absence (○) of blockers or following exposure to scorpion venom (SV, 8.7 μg ml^{-1}; ●) or combined SV, N^G-monomethyl-L-arginine (L-NMMA, 350 μM) and oxyhaemoglobin (HbO, 20 μM, △). The ACh response in the combined presence of SV, L-NMMA and HbO was nearly abolished. (b) Concentration-relaxation relationship for Cys-NO in the absence (□, $n = 4$) or presence (■) of SV. SV had no significant effect on this relationship. Shown are mean values ± s.e.

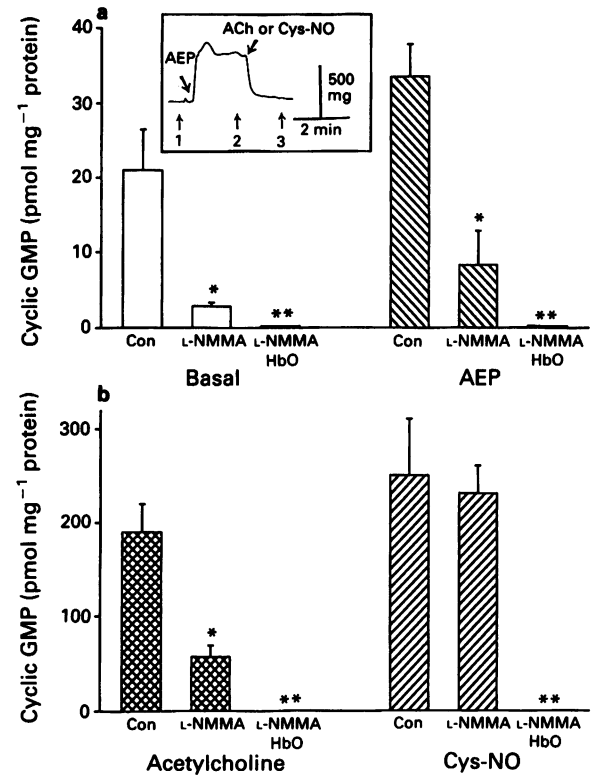

Figure 4 Cyclic GMP accumulation in guinea-pig coronary artery. Inset: Diagram of the conditions under which tissue cyclic GMP measurements are made. Shown is a representative contractile response to 2-(2-aminoethyl)pyridine (AEP, 100 μM) followed by relaxation with acetylcholine (ACh, 0.35 μM). Arrow (1) Basal GMP. These measurements were made in the absence of agonist on tissues equilibrated as described for other contractile experiments. Arrows (2) cyclic GMP during contraction. These measurements were made during peak contraction at an average time of 1.9 ± 0.1 min ($n = 15$) after addition of AEP. Arrow (3) cyclic GMP during relaxation. These measurements were made 1.3 ± 0.1 min ($n = 31$) after addition of either ACh or L-nitrosocysteine (Cys-NO) in the continued presence of AEP. All cyclic GMP measurements were made on endothelium intact vessel segments. (a) and (b) Cyclic GMP levels obtained either in the absence of blockers of the NO pathway (Con), in the presence of N^G-monomethyl-L-arginine (L-NMMA, 350 μM) or in the presence of combined L-NMMA and oxyhaemoglobin (HbO, 20 μM). L-NMMA significantly decreased cyclic GMP levels in basal, AEP and ACh tissues ($P < 0.05$) (a). In the presence of combined L-NMMA and HbO, cyclic GMP levels never exceeded 0.2 pmol mg^{-1} protein (i.e. less than 1% of basal control values) in all four experimental conditions. Asterisks indicate values which are significantly different from the control value of each group. Shown are mean values ± s.e.

The rise in cyclic GMP levels observed with ACh is probably due to release of NO. To provide direct evidence for this conclusion, additional experiments were undertaken in which NO synthase activity was inhibited with L-NMMA (350 μM, 30 min). In the presence of L-NMMA both the basal levels of cyclic GMP ($n = 4$) as well as the levels measured during contraction with AEP ($n = 5$) were significantly reduced. In addition, the ACh-induced increase in cyclic GMP was reduced 66% ($n = 5$) by L-NMMA. In contrast, there was no significant difference in the levels of cyclic GMP reached with Cys-NO in the presence ($n = 5$) or absence of L-NMMA (Figure 4). There was also no significant difference in the rise in cyclic GMP produced with ACh in the presence or absence of SV (8.7 μg ml^{-1}, $n = 4$, data not shown).

Although L-NMMA significantly reduced the rise in cyclic GMP levels elicited with ACh it did not abolish this action. To determine whether the L-NMMA resistant rise in cyclic GMP was due to incomplete blockade of NO synthase or to another mechanism, additional experiments were undertaken with combined L-NMMA (350 μM) and HbO (20 μM). Basal cyclic GMP levels in the presence of combined L-NMMA and HbO (30 min) were reduced to less than 1% of control ($n = 6$) and there was no significant increase in cyclic GMP levels upon application of either AEP ($n = 6$), ACh ($n = 5$) or Cys-NO ($n = 6$) (Figure 4). Since relaxation was still observed with ACh in the presence of combined L-NMMA and HbO our data suggest that a portion of the ACh-induced relaxation is independent of either NO or changes in cyclic GMP levels in the tissue.

Intracellular experiments

Comparison of the effects of acetylcholine and L-nitrosocysteine on membrane potential The results obtained from contractile experiments and measurements of cyclic GMP levels suggest that the actions of ACh are not identical to Cys-NO. To distinguish further between these two agents their effects on membrane potential were measured. The mean resting membrane potential of guinea-pig coronary artery cells was − 56 mV. Application of ACh (0.35 μM) hyperpolarized cells by 20 mV. In contrast, Cys-NO (3.5 μM) gave rise to a mean hyperpolarization of only 3.5 mV. Examples of these changes in membrane potential are shown in Figure 5 and results are summarized in Table 1.

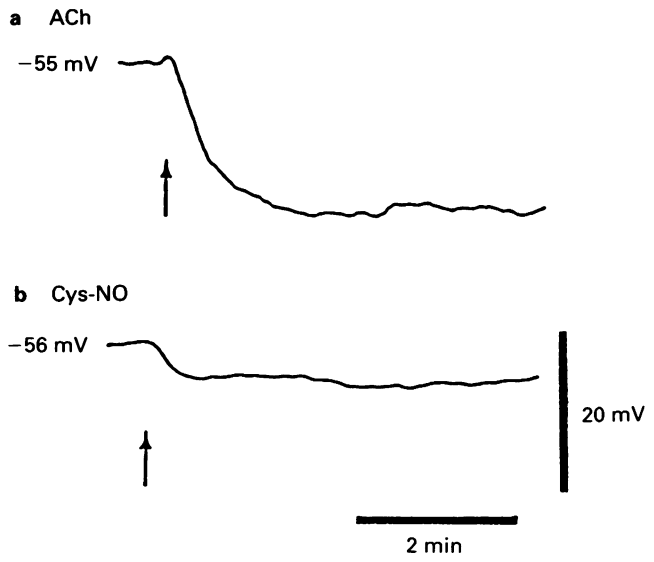

Figure 5 Representative examples of the effect of acetylcholine (ACh, 0.35 μM) and L-nitrosocysteine (Cys-NO, 3.5 μM) on resting membrane potential in two different cells in a segment of guinea-pig coronary artery. Each drug was added at the arrow followed by its respective hyperpolarization (downward deflection).

The effects of ACh and Cys-NO on membrane potential were also measured in tissues exposed to the contractile agonist AEP. AEP (100 μM) depolarized cells from a mean of − 56 mV to − 42 mV. Once a plateau of depolarization was reached either ACh or Cys-NO was included in the superfusate. ACh reversed the depolarization produced by AEP and hyperpolarized the tissue below the control level so that the total change in membrane potential with ACh in the presence of AEP (i.e., 33 mV) was greater than the change in membrane potential in the absence of AEP (i.e., 20 mV). In contrast, Cys-NO increased the polarization of the membrane by only 5 mV (see Table 1). This change in membrane potential was not significantly different from the change observed in the absence of AEP. In spite of the large difference in the effects of ACh and Cys-NO on membrane potential both of these agents relaxed tissues by at least 90%.

Effect of N^G-monomethyl-L-arginine and oxyhaemoglobin on acetylcholine-induced hyperpolarization In the presence of combined HbO and L-NMMA, ACh failed to produce a significant increase in cyclic GMP levels suggesting that this combination of drugs produces complete blockade of the NO pathway (Figure 4). However, under the same conditions there was only a small shift in the concentration-relaxation relationship for ACh. The relaxation which remains in the absence of an increase in cyclic GMP levels in the tissue is likely to be due to release of EDHF from the endothelium. To provide direct evidence for this hypothesis intracellular responses to ACh were measured in the presence of either L-NMMA (350 μM) or L-NMMA plus HbO (20 μM). A control response to ACh (0.35 μM) was obtained followed by a 15 min re-equilibration period in normal Krebs solution. The tissue was then superfused with L-NMMA or L-NMMA plus HbO. The resting membrane potential following 30 min exposure to either L-NMMA alone or L-NMMA plus HbO was not significantly different from control nor was there a significant reduction in the amplitude of hyperpolarization with ACh (Table 2). These data suggest that the ACh-

Table 1 Effect of acetylcholine (ACh) and L-nitrosocysteine (Cys-NO) on membrane potential in the presence and absence of 2-(2-aminoethyl)pyridine (AEP)

	E_m (mV)	n
Control	− 56 ± 1	45
	ΔE_m (mV)	n
ACh (0.35 μM)	20 ± 1	10
Cys-NO (3.5 μM)	3.5 ± 1	3
	E_m (mV)	n
AEP (100 μM)	− 42 ± 4	7
	ΔE_m (mV)	n
AEP + ACh (0.35 μM)	33 ± 6	4
AEP + Cys-NO (3.5 μM)	5 ± 3	3

E_m, membrane potential
ΔE_m (mV), change in E_m with the drugs indicated.

Table 2 Effect of acetylcholine (ACh) on membrane potential following blockade of the NO pathway

	E_m (mV)	*ACh* (0.35 μM) *hyperpolarization*	n
Control	− 56 ± 2	16 ± 1	8
L-NMMA (350 μM)	− 52 ± 2	16 ± 1	5
LNMMA + HbO (20 μM)	− 53 ± 3	17 ± 3	3

induced hyperpolarization is generated, in large part, by an NO-independent pathway.

Discussion

ACh elicits endothelium-dependent relaxation and hyperpolarization in the guinea-pig coronary artery (Kitamura & Kuriyama, 1979; Keef & Bowen, 1989; Chen *et al.*, 1991). The present study indicates that ACh also significantly increases cyclic GMP levels in this blood vessel. Our results suggest that the effects of ACh are due to the release of two distinctly different factors from the endothelium, namely, NO and EDHF. Although each factor is capable of producing complete relaxation of the tissue, the pathway by which they produce relaxation appears to differ significantly. Specifically, the NO pathway leads to an increase in tissue cyclic GMP levels and is relatively independent of changes in membrane potential whereas the EDHF pathway leads to membrane hyperpolarization and is independent of changes in cyclic GMP levels in the tissue.

The coronary artery exhibited significant basal levels of cyclic GMP. These levels were reduced to 12% of control with the arginine analogue by L-NMMA and to less than 1% of control with combined L-NMMA and HbO. The results suggest that basal cyclic GMP levels are almost exclusively due to spontaneous release of NO. The H_1 receptor agonist, AEP, was capable of eliciting contraction in the presence of these basal levels of cyclic GMP and interestingly, although H_1 receptors are also reported to be present on endothelial cells in some blood vessels (Chen & Suzuki, 1989; Malinowska & Schlicker, 1993) we did not observe a significant change in cyclic GMP levels when AEP was added to the tissue. Basal cyclic GMP levels appear to modulate contractions however, since significantly larger contractions were obtained in the presence of either L-NMMA or HbO.

To investigate the contribution of NO to the relaxation induced with ACh, experiments were undertaken with blockers of the NO pathway. L-NMMA did not shift the ACh concentration-relaxation relationship whereas some shift to the right was observed with combined HbO and L-NMMA. The results with L-NMMA alone are of particular interest since in some blood vessels this concentration of L-NMMA has been reported to produce almost complete abolition of the ACh-induced relaxation (e.g., rat aorta, Rees *et al.*, 1990). One possible explanation for the difference is that relaxation with ACh in the rat aorta is primarily due to NO whereas in the guinea-pig coronary artery EDHF also contributes significantly to relaxation. In fact, the limited effect which combined L-NMMA and HbO have on the ACh-induced relaxation relationship in guinea-pig coronary artery might lead one to speculate that only small quantities of NO are released upon exposure of the guinea-pig coronary artery to ACh. This is apparently not the case however since concentrations of ACh and Cys-NO which produced equivalent relaxation also produced equivalent increases in cyclic GMP levels and in both cases the rise in cyclic GMP was abolished with combined L-NMMA and HbO. The results therefore suggest that significant quantities of NO are released upon addition of ACh to the coronary artery. The fact that relaxation is only minimally reduced when the NO pathway is blocked must therefore be attributed to the actions of a second factor. The importance of both NO and EDHF to relaxation is further underscored by that fact that when either the NO or EDHF pathway is separately blocked only a small shift in the ACh concentration-relaxation relationship is observed whereas when both pathways are blocked almost complete abolition of relaxation is obtained.

The ACh-induced hyperpolarization was not significantly reduced in the presence of combined L-NMMA and HbO, suggesting that this response is largely dependent upon release of EDHF. A similar conclusion has been reached by others using various blockers of the NO pathway (Komori *et al.*, 1988; Chen *et al.*, 1988; Huang *et al.*, 1988; Nishiye *et al.*, 1989; Brayden, 1990; Nagao & Vanhoutte, 1991; Chen *et al.*, 1991; Garland & McPherson, 1992). Since combined L-NMMA and HbO entirely abolished the rise in cyclic GMP elicited with ACh our results also provide direct evidence that the EDHF induced hyperpolarization is independent of cyclic GMP. Interestingly, we observed that Cys-NO was also capable of producing a small but significant increase in membrane polarization. Nitric oxide induced hyperpolarization has been reported in some blood vessels (Garland & McPherson, 1992; Tare *et al.*, 1991; Kitamura *et al.*, 1993) but is absent in others (Bény & Brunet, 1988; Brayden, 1990; Brayden *et al.*, 1991; Komori *et al.*, 1988). Since the amplitude of the ACh-induced hyperpolarization before and after addition of combined L-NMMA and HbO in the guinea-pig coronary artery was not significantly different it suggests either that the NO which is released upon addition of ACh does not reach a concentration that is sufficient to affect membrane potential or alternatively that the actions of NO and EDHF on membrane potential combined in a manner which is less than additive so that removing the NO pathway does not lead to a detectable reduction in the ACh-induced hyperpolarization. Our experiments cannot distinguish between these two possibilities.

The hyperpolarization which occurs with ACh is likely to be due to the opening of K^+ channels in the smooth muscle membrane as previously suggested (Kitamura & Kuriyama, 1979; Brayden, 1990; Chen *et al.*, 1991; Eckman *et al.*, 1992; Garland & McPherson, 1992). In the rabbit basilar artery the ACh-induced hyperpolarization appears to involve the opening of ATP-dependent K^+ channels (Brayden *et al.*, 1991). However, in several other blood vessels including the guinea-pig coronary artery this does not appear to be the case since membrane hyperpolarization in these tissues is not blocked with glibenclamide, an antagonist of ATP-dependent K^+ channels (e.g., McPherson & Angus, 1991; Eckman *et al.*, 1992; for review see Edwards & Weston, 1993). Regardless of the K^+ channel involved, the relaxation induced by EDHF is likely to involve hyperpolarization-induced closing of voltage-dependent Ca^{2+} channels (Weir & Weston, 1986; Meisheri *et al.*, 1988; Standen *et al.*, 1989; Brayden *et al.*, 1991).

The most effective blocker of ACh-induced hyperpolarization in the guinea-pig coronary artery which we have observed to date is crude scorpion venom (SV). In a previous study we reported that this venom reduced the hyperpolarization elicited with ACh by 94% (Eckman *et al.*, 1992). Scorpion venom contains a number of different K^+ channel blockers (Strong, 1990) and we hypothesize that the effects of SV on hyperpolarization are due to the actions of one or more of these blockers on smooth muscle K^+ channels. Another possibility which cannot be excluded given the present results is that SV specifically antagonizes the synthesis or release of EDHF. However, in spite of this limitation, several lines of evidence suggest that the effects of SV are relatively specific for the EDHF pathway: (1) SV did not reduce the relaxant responses to Cys-NO indicating that the smooth muscle cells are still capable of relaxing and specifically of relaxing in response to NO; (2) SV reduced but did not abolish relaxations elicited with ACh indicating that some factor is still released from the endothelium in the presence of SV; (3) the relaxation which remains in the presence of SV is almost completely abolished when blockers of the NO pathway are also included suggesting that the relaxation which occurs in the presence of SV is due to NO. Point (3) is further supported by the observation that: (4) SV does not block the increase in cyclic GMP produced by ACh.

The relationship of NO and EDHF to the physiological control of coronary blood flow is still uncertain. Blood flow to the heart depends in large part upon local factors which serve to match the flow of blood to the work of the heart (Parratt, 1982). The precise mechanism of this matching is unknown but the endothelium may play an important role (Bigaud & Vatner, 1991). Our results suggest that the cor-

onary endothelium releases two distinctly different factors that are each capable of producing significant relaxation of the adjacent smooth muscle. Since the mechanism by which these two factors produce relaxation differ, their contribution to relaxation *in vivo* may also differ depending upon the circumstances which have led to contraction. If contraction involved the opening of voltage-dependent Ca^{2+} channels, then EDHF would be predicted to be an effective vasodilator. In contrast, under circumstances in which contraction involved a minimal change in Ca^{2+} entry (e.g., contraction induced with phorbol ester) NO would be predicted to be the more effective vasodilator. Since the chemical nature of 'EDHF' is not yet known, it is also possible that the distance over which NO and EDHF exert an effect could differ from one another or that their synthesis and release could be differentially regulated *in vivo*. Thus, although both NO and EDHF relax the coronary artery, the differences seen between these two factors may increase the effectiveness of regulating coronary blood flow under diverse metabolic conditions.

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