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Endothelium-dependent relaxation resistant to N^G-nitro-L-arginine in rat aorta

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Abstract

Experiments were designed to determine whether cyclic GMP-independent relaxation is involved in the endothelium-dependent vascular relaxation response of rat aortic strip to acetylcholine. The relaxation response to acetylcholine in the presence of 3×10^{-4} M N^G-nitro-L-arginine was apparent when the precontraction was induced by norepinephrine at 5×10^{-9} M or 10^{-8} M. The relaxation response to acetylcholine resistant to N^G-nitro-L-arginine was abolished by 10^{-6} M atropine, 10 mM tetraethylammonium, or endothelium removal, but was not inhibited by 10^{-5} M indomethacin, 3×10^{-6} M oxyhemoglobin or 10^{-5} M glibenclamide. The response was virtually abolished when the vascular strips had been preconstricted with 20 mM KCl. The increase in vascular cyclic GMP levels induced by 10^{-5} M acetylcholine was completely abolished by 3×10^{-4} M N^G-nitro-L-arginine. These results suggest that acetylcholine-induced endothelium-dependent relaxation resistant to N^G-nitro-L-arginine in rat aorta is unmasked when the precontractile force is caused by lower concentrations of norepinephrine and the relaxation is mediated by a cyclic GMP-independent mechanism, possibly an endothelium-derived hyperpolarizing factor.

Keywords: Endothelium; N^G-Nitro-L-arginine; EDHF (endothelium-derived hyperpolarizing factor); Acetylcholine; Aorta; (Rat)

1. Introduction

Acetylcholine causes relaxation of vascular smooth muscle cells by releasing endothelium-derived relaxing factor (EDRF) in a variety of blood vessels (Furchgott and Zawadzki, 1980; Furchgott, 1983). Nitric oxide (Ignarro et al., 1987a,b; Palmer et al., 1987) or a closely related nitroso compound (Myers et al., 1990) is considered to be the most likely candidate for EDRF. Although L-arginine analogues, inhibitors of EDRF synthesis, such as N^G-monomethyl-L-arginine or N^G-nitro-L-arginine, virtually abolish basal and stimulated EDRF release from cultured endothelial cells (Palmer et al., 1988; Ishii et al., 1990), they impair but do not abolish agonist-stimulated relaxation in isolated rabbit aorta (Palmer et al., 1988; Moore et al., 1990), pig coronary artery (Richard et al., 1990) and bovine intrapulmonary artery (Gold et al., 1990) even at high concentrations. Thus, incomplete inhibition by the inhibitors suggests that EDRF is not critical for complete endothelium-dependent relaxation and that a relaxing substance(s) other than EDRF may play a role in the relaxation. Electrophysiological studies have revealed that vascular endothelial cells can release an as yet unidentified hyperpolarizing factor in response to vasodilators, which is called the endothelium-derived hyperpolarizing factor (EDHF) (Taylor and Weston, 1988). Hyperpolarization has been shown to accompany endothelium-dependent relaxation in response to bradykinin in pig coronary artery (Bény and Brunet, 1988a) and to acetylcholine in guinea pig (Keef and Bowen, 1989) and dog coronary artery (Feletou and Vanhoutte, 1988; Chen et al., 1989; Hoeffner et al., 1989), dog mesenteric artery (Komori et al., 1988), and rat pulmonary artery and aorta (Chen et al., 1988; Taylor et al., 1988; Chen and Suzuki, 1989). Thus, hyperpolarization is most likely to be associated with a relaxation response. The relaxation response resistant to L-arginine analogues in pig coronary artery has been reported to be mediated by a cyclic GMP (cGMP)-independent mechanism, possibly by EDHF (Cowan and

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Cohen, 1991; Nagao and Vanhoutte, 1992). However, in rat aorta, acetylcholine produced endothelium-dependent hyperpolarization (Chen et al., 1988; Chen and Suzuki, 1989) although the relaxation response to acetylcholine was completely inhibited by higher concentrations of L-arginine analogues (Rees et al., 1990; Thomas and Ramwell, 1991; Vargas et al., 1991). Thus, this finding in rat aorta suggests that relaxing substance(s) other than EDRF, including EDHF, may not be involved in the endothelium-dependent relaxation response to acetylcholine.

The aim of the present study was to determine whether a relaxation response that is not mediated by EDRF contributes to the relaxation response to acetylcholine in rat aorta.

2. Materials and methods

2.1. Tissue preparation

Thoracic aortas were obtained from male Wistar rats, weighing 250–300 g, after their decapitation. The aortas were cut helically into vascular strips (2.0 mm wide, 15 mm long) and set up isometrically in vitro, as previously described (Altura and Altura, 1970). The strips were fixed vertically between hooks in a 10-ml organ bath containing Krebs-Ringer solution, which was maintained at 37°C, pH 7.4, and aerated with a mixture of 95% O_2 and 5% CO_2. The Krebs-Ringer solution was composed of (mM):NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; glucose, 10; $NaHCO_3$, 25.

2.2. Measurement of tension

Isometric tension, monitored with a force-displacement transducer (Nihon Kohden Kohgyo Co., Tokyo, Japan) to which the upper ends of the strips were connected, was recorded with a pen recorder (Nihon Kohden Kohgyo Co.). The artery strips were equilibrated for approximately 2 h, during which time the medium was replaced every 15 min. The strips were adjusted for a resting tension of 1.0 g. The endothelium was removed from some strips by rubbing the intimal surface with filter paper (Furchgott and Zawadzki, 1980). Successful removal of the endothelial cells was confirmed by the inability of 10^{-6} M acetylcholine to induce relaxation and by histological examination of the intimal surface using a silver-staining technique (Poole et al., 1958). After the precontraction with norepinephrine or KCl had reached its peak tension, acetylcholine or papaverine was added cumulatively to the organ bath. In an experiment with various inhibitors, intact arterial strips were first incubated for 10 min with atropine or for 60 min with N^G-nitro-L-arginine, indomethacin, tetraethylammonium, oxyhemoglobin or glibenclamide before the precontraction with norepinephrine. Relaxation was expressed as the percentage decrease in tension from contraction in response to norepinephrine.

2.3. Preparation of oxyhemoglobin

Bovine hemoglobin type 1, containing a mixture of oxyhemoglobin and the oxidized derivative, methemoglobin, was purchased from Sigma Chemical Co. (St. Louis, MO). Oxyhemoglobin was prepared by reducing methemoglobin, as previously reported (Martin et al., 1985). Briefly, a 10-fold molar excess of the reducing agent, sodium dithionate, was added to 1 mM solutions of hemoglobin and then the sodium dithionate was removed by dialysis against 100 volumes of distilled water for 2 h at 4°C. The hemoglobin solutions were frozen in aliquots at −80°C and stored for up to 14 days.

2.4. Measurement of cGMP levels

cGMP measurements were carried out using vascular strips which had been mounted in the organ bath and equilibrated for 2 h, as described above. The vascular strips were incubated with N^G-nitro-L-arginine for 60 min and then were stimulated with 10^{-5} M acetylcholine for 1 min, which evoked an increase in cGMP production, as previously described (Rapoport and Murad, 1983; Furchgott et al., 1984) or for 3 min, which led to the maximal relaxation response to 10^{-5} M acetylcholine in the presence of N^G-nitro-L-arginine. The strips were immediately frozen in liquid nitrogen, then homogenized in 6% trichloroacetic acid and centrifuged for 15 min at $3000 \times g$. Supernatant fractions were extracted four times in 5 volumes of water-saturated ether and then evaporated to dryness. These extracts were stored at −80°C until assay of the cGMP. The extracts were reconstituted in sodium acetate buffer (50 mM at pH 6.2) containing theophylline (1 mM) and then were acetylated. cGMP levels were determined by radioimmunoassay using New England Nuclear Kits (Boston, MA, USA), as previously described (Ignarro et al., 1981). The tissue residue was dissolved in 2 M NaOH and protein content was determined using a dye-binding assay (Bio-rad), with bovine serum albumin as the standard. The concentration of cGMP was expressed as pmol/mg protein.

2.5. Drugs

l-Norepinephrine hydrochloride, acetylcholine chloride, papaverine hydrochloride, atropine sulfate, indomethacin, tetraethylammonium chloride, glibenclamide and sodium dithionate were obtained from

Sigma Chemical Co. (St. Louis, MO). N^G-nitro-L-arginine was obtained from Wako Pure Chemical Industries (Tokyo, Japan). Indomethacin was dissolved in an equimolar concentration of Na_2CO_3. Glibenclamide was dissolved in 100% ethanol. Neither Na_2CO_3 nor ethanol affected the responses of the tissues at the concentrations used. Ascorbic acid (0.1 mM) was present in all experiments with norepinephrine. All other drugs were dissolved in distilled water. The drugs were added to the organ bath medium at 50–100 μl; drug concentrations are reported as the final molar concentration (in M) in the bath.

2.6. Calculations and statistical analysis

The results are expressed as means ± S.E.M. The concentration needed to reach 50% maximal relaxation (ED_{50} values) was determined graphically from the linear regression of the 20–80% region of the log concentration-response curves. The statistical significance of the observed differences was tested with Student's unpaired *t*-test or the analysis of variance followed by Scheffé's test (for multiple comparisons). The level of significance was $P < 0.05$.

3. Results

3.1. Vasorelaxation response to acetylcholine resistant to N^G-nitro-L-arginine.

Fig. 1a shows the relationship between various concentrations of norepinephrine (5×10^{-9}–10^{-7} M) used to cause precontraction in the presence of N^G-nitro-L-arginine at 5×10^{-5} M and the degree of the relaxation response to acetylcholine. With norepinephrine at 5×10^{-8} M and 10^{-7} M, the relaxation response to acetylcholine was not observed, but when the vascular strips had been precontracted with norepinephrine at 5×10^{-9} M or 10^{-8} M, acetylcholine caused significant relaxation responses, with a greater response for the strips precontracted with 5×10^{-9} M than with 10^{-8} M norepinephrine. Fig. 1b shows the acetylcholine-induced relaxation response of the vascular strips precontracted with 5×10^{-9} M norepinephrine in the presence of five concentrations of N^G-nitro-L-arginine (5×10^{-5}, 1, 3, 5×10^{-4} and 10^{-3} M). The relaxation response to acetylcholine was greater at 5×10^{-5} M N^G-nitro-L-arginine than at the other four concentrations, suggesting that the EDRF-mediated relaxation had not been sufficiently blocked at this concentration. Concentrations of N^G-nitro-L-arginine at 10^{-4} M or higher did not produce significantly more antagonism of the relaxation in response to acetylcholine. In further experiments, we therefore used N^G-nitro-L-arginine at 3×10^{-4} M to completely block the relaxation response due to EDRF synthesis from L-arginine stimulated by acetylcholine, and norepinephrine at 5×10^{-9} M to elicit a clear relaxation response to acetylcholine in the presence of N^G-nitro-L-arginine. The precontractile level induced by 5×10^{-9} M norepinephrine was significantly augmented approximately five-fold (1.39 ± 0.14 g tension, $n = 7$) in the presence of 3×10^{-4} M N^G-nitro-L-arginine, as compared to that in its absence (0.26 ± 0.06 g tension, $n = 7$). This precontractile level was similar to that induced by 10^{-7} M norepinephrine in the absence of the inhibitor (1.35 ± 0.12 g tension, $n = 7$). The precontraction remained at a stable peak tension for at least 15 min. The threshold concentration of acetyl-

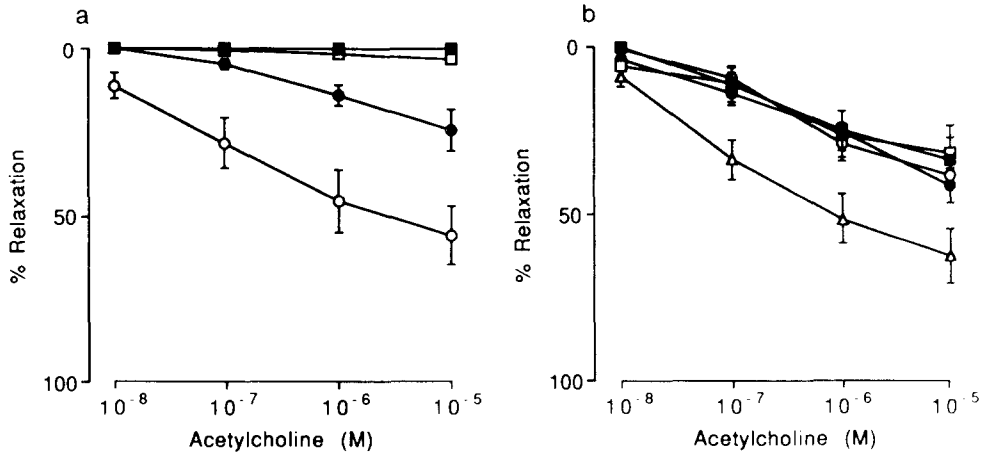

Fig. 1. Vasorelaxant effect of acetylcholine in rat aorta: (a) shows the relaxation response to acetylcholine in the strips precontracted with 5×10^{-9} M (○), 10^{-8} M (●), 5×10^{-8} M (□) or 10^{-7} M (■) norepinephrine in the presence of 5×10^{-5} M N^G-nitro-L-arginine; (b) shows the relaxation response to acetylcholine in the strips precontracted with 5×10^{-9} M norepinephrine in the presence of 5×10^{-5} M (△), 10^{-4} M (○), 3×10^{-4} M (●), 5×10^{-4} M (□) or 10^{-3} M (■) N^G-nitro-L-arginine. Each point represents the mean of seven experiments with S.E.M. shown by vertical bars.

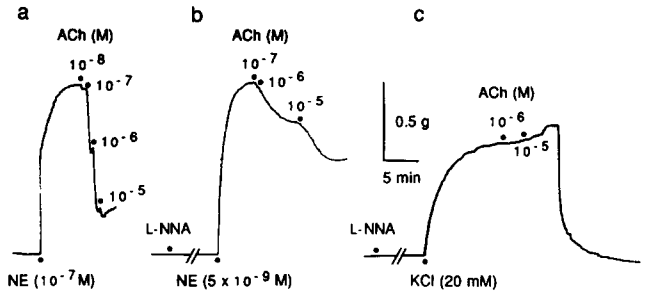

Fig. 2. Representative traces showing the relaxation response to acetylcholine (ACh) in rat aortic strips precontracted with (a) 10^{-7} M norepinephrine (NE) in the absence of N^G-nitro-L-arginine (L-NNA) or with (b) 5×10^{-9} M NE and (c) 20 mM KCl in the presence of 3×10^{-4} M L-NNA.

choline for the relaxation response was 10^{-7} M, and acetylcholine produced a slowly developing and sustained relaxation with a lag time of approximately 20 s to its start in the presence of N^G-nitro-L-arginine when compared with that in its absence (Fig. 2a,b). The relaxation resistant to N^G-nitro-L-arginine was completely blocked in the vascular strips precontracted with 20 mM KCl (Fig. 2c, $n = 7$).

3.2. Effects of various inhibitors or endothelium removal on the relaxation resistant to N^G-nitro-L-arginine

Fig. 3a and b show the effect of 10^{-6} M atropine, 10^{-5} M indomethacin, 3×10^{-6} M oxyhemoglobin or endothelium removal on acetylcholine-induced vasorelaxation resistant to 3×10^{-4} M N^G-nitro-L-arginine. This response was completely abolished by atropine or removal of the endothelium, but was not affected by indomethacin or oxyhemoglobin. Also, the resistant relaxation was completely inhibited by tetraethylammonium but not by glibenclamide (Fig. 3c).

3.3. Relaxation response to papaverine in the presence of N^G-nitro-L-arginine or acetylcholine in the presence of tetraethylammonium in vascular strips contracted to equivalent precontractile tensions

There were no significant differences in the relaxation responses to papaverine between the vascular strips precontracted with 10^{-7} M norepinephrine in the absence of N^G-nitro-L-arginine and with 5×10^{-9} M norepinephrine in the presence of 3×10^{-4} M N^G-nitro-L-arginine, under the two experimental conditions in which the precontractile tensions were similar (see legend to Fig. 4a). Fig. 4b shows the inhibitory effect of tetraethylammonium on acetylcholine-induced relaxation in the absence of N^G-nitro-L-arginine. Tetraethylammonium (10 mM) enhanced the contractile response of the vascular strips to norepinephrine in such a way that the concentration of norepinephrine required for the organ bath to raise the tone to a level equivalent to that seen before tetraethylammonium addition had to be decreased from 10^{-7} M to 3×10^{-8} M (see legend to Fig. 4b). The papaverine-induced relaxation did not differ significantly between the vascular strips precontracted with norepinephrine in the presence of 10 mM tetraethylammonium (ED_{50}, $9.7 \pm 0.6 \times 10^{-7}$ M; maximal relaxation, $98.5 \pm 3.8\%$, $n = 6$) or 20 mM KCl (ED_{50}, $9.5 \pm 0.5 \times 10^{-7}$ M; maximal relaxation, $96.7 \pm 5.5\%$, $n = 6$) and in their absence (ED_{50}, $9.0 \pm 0.6 \times 10^{-7}$ M; maximal relaxation, $96.8 \pm 4.7\%$, $n = 6$). The relaxation response to acetylcholine was inhibited by tetraethylammonium but a mean maximal relaxation of 52.4% remained. In the presence of

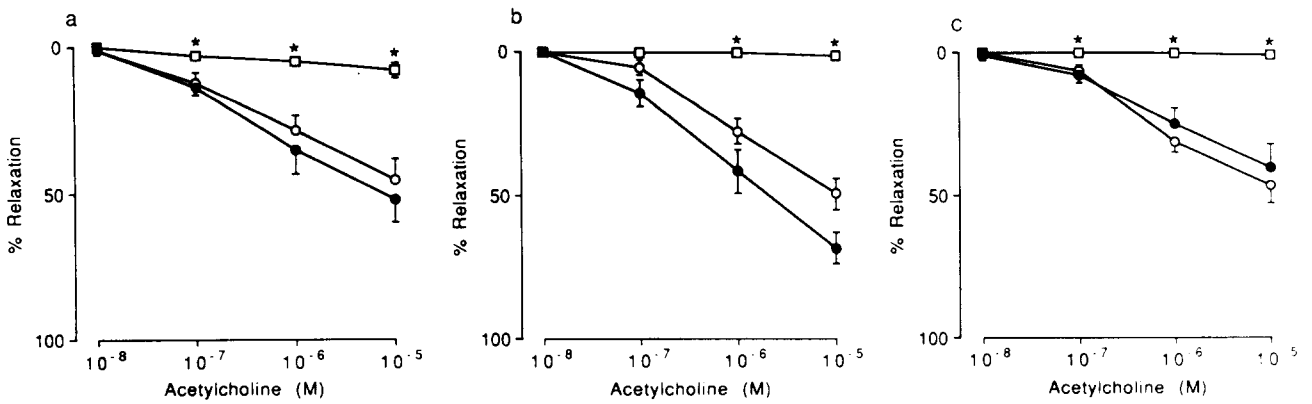

Fig. 3. Effects of various treatments on the relaxation of rat aortic strips induced by acetylcholine in the presence of 3×10^{-4} M N^G-nitro-L-arginine: (a) shows the effect of 10^{-6} M atropine (□) or 10^{-5} M indomethacin (●); (b) shows the effect of 3×10^{-6} M oxyhemoglobin (●) or endothelium removal (□); and (c) shows the effect of 10^{-5} M glibenclamide (●) or 10 mM tetraethylammonium (□). The symbol (○) in (a), (b) and (c) shows the relaxation response to acetylcholine in the absence of treatment (control). The vascular strips were precontracted with 5×10^{-9} M norepinephrine. Each point represents the mean of seven experiments with S.E.M. shown by vertical bars. * Significantly different ($P < 0.01$) from control value.

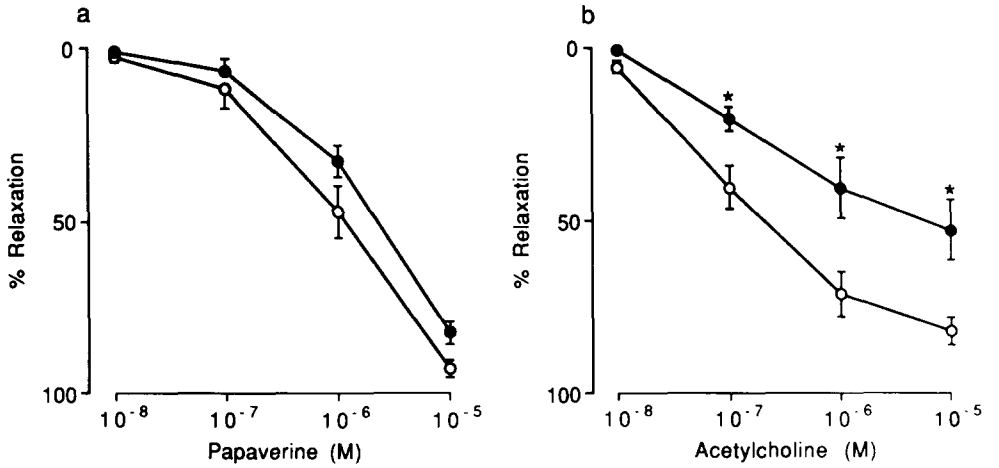

Fig. 4. Concentration-response curves for the relaxation induced by (a) papaverine and (b) acetylcholine in rat aortic strips contracted to the equivalent precontractile tensions: (a) shows the relaxation response to papaverine in the presence of 3×10^{-4} M N^G-nitro-L-arginine (●) or in its absence (control, ○). The vascular strips were precontracted with 5×10^{-9} M norepinephrine in the presence of N^G-nitro-L-arginine (contraction level, 1.37 ± 0.15 g tension) and with 10^{-7} M norepinephrine in the control (contraction level, 1.32 ± 0.09 g tension): (b) shows the vasorelaxant effect of acetylcholine in the presence of 10 mM tetraethylammonium (●) and in the absence of tetraethylammonium (control, ○). The vascular strips were precontracted with 3×10^{-8} M norepinephrine in the presence of tetraethylammonium (contraction level, 1.45 ± 0.11 g tension) and with 10^{-7} M norepinephrine in the absence of tetraethylammonium (contraction level, 1.33 ± 0.11 g tension). Each point represents the mean of seven to nine experiments with S.E.M. shown by vertical bars. * Significantly different ($P < 0.01$) from control values.

tetraethylammonium (ED_{50}, $2.7 \pm 0.3 \times 10^{-7}$ M; maximal relaxation, $53.4 \pm 3.5\%$, $n = 6$), this acetylcholine-induced relaxation was further inhibited by 10 mM KCl (ED_{50}, $4.5 \pm 0.2 \times 10^{-7}$ M; maximal relaxation, $42.5 \pm 4.6\%$, $n = 6$, $P < 0.05$ for both values).

3.4. Effect of N^G-nitro-L-arginine on cGMP formation

Fig. 5 shows the effect of N^G-nitro-L-arginine on cGMP formation. N^G-nitro-L-arginine significantly reduced the basal content of cGMP in rat aortic strips with endothelium (from 0.81 ± 0.15 to 0.35 ± 0.05 pmol/mg protein). Acetylcholine (10^{-5} M, 1 min) enhanced the tissue content of cGMP about six-fold (4.84 ± 0.22 pmol/mg protein) when compared with the basal values. The production of cGMP evoked by acetylcholine was significantly inhibited by 3×10^{-4} M N^G-nitro-L-arginine (0.40 ± 0.05 pmol/mg protein). Furthermore, acetylcholine did not cause an increase in cGMP at 3 min (0.33 ± 0.05 pmol/mg protein) after the addition, when the relaxation induced by 10^{-5} M acetylcholine reached its plateau level.

4. Discussion

In rat aorta, acetylcholine caused an endothelium-dependent relaxation that did not exceed 40% in the presence of methylene blue, a guanylate cyclase inhibitor, and produced endothelium-dependent hyperpolarization which was not blocked by methylene blue (Chen and Suzuki, 1989). It has been suggested that EDHF is responsible for about 20–40% of the acetylcholine-induced endothelium-dependent relaxation in rat aorta, which is less than that caused by EDRF (Chen and Suzuki, 1989). However, when L-arginine analogues were used instead of methylene blue in rat

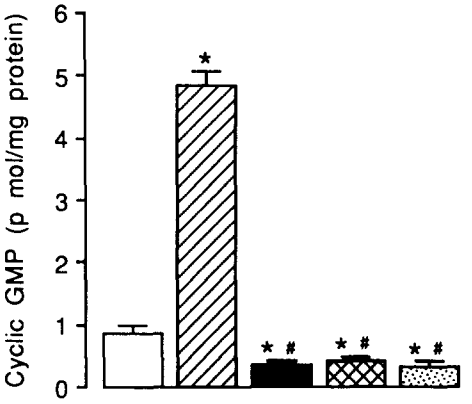

Fig. 5. Effects of N^G-nitro-L-arginine on acetylcholine-induced production of cyclic GMP in rat aortic strips with endothelium. The vascular strips were incubated with 3×10^{-4} M N^G-nitro-L-arginine for 60 min. Open column shows the control value obtained without treatment. Hatched column shows the values obtained 1 min after the addition of 10^{-5} M acetylcholine in the absence of N^G-nitro-L-arginine. Solid, cross-hatched and stippled columns show the values obtained with N^G-nitro-L-arginine alone, 1 min and 3 min after the addition of 10^{-5} M acetylcholine in the presence of N^G-nitro-L-arginine, respectively. * Significantly different ($P < 0.01$) from control value. # Significantly different ($P < 0.01$) from the values obtained in the presence of acetylcholine in the absence of N^G-nitro-L-arginine. Columns represent the mean of eight to nine experiments with S.E.M. shown by vertical bars.

aorta, the acetylcholine-induced relaxation was completely abolished (Rees et al., 1990; Thomas and Ramwell, 1991; Vargas et al., 1991; Nagao et al., 1992). Thus, the relationship between acetylcholine-induced relaxation and EDHF is equivocal.

In the present study, in the vascular strips precontracted with norepinephrine concentrations of 5×10^{-8} M or higher in the presence of N^G-nitro-L-arginine, acetylcholine did not elicit a relaxation response. However, when lower concentrations of norepinephrine (5×10^{-9} M or 10^{-8} M) were used, the relaxation was apparent. It was blocked by removal of the endothelium or atropine. These results suggest that the relaxation response is endothelium-dependent and is mediated via stimulation of a muscarinic receptor. The relaxation response to acetylcholine resistant to the inhibition of EDRF synthesis by N^G-nitro-L-arginine is most likely to be produced by an EDRF-independent mechanism for the following reasons. First, incomplete inhibition by N^G-nitro-L-arginine (3×10^{-4} M) of the pathway of EDRF synthesis from L-arginine is unlikely, as even a massive concentration (1 mM) caused no further inhibition of the N^G-nitro-L-arginine-resistant relaxation. Furthermore, N^G-nitro-L-arginine is more potent than N^G-methyl-L-arginine and N^G-amino-L-arginine as a specific inhibitor of EDRF synthesis (Ishii et al., 1990; Moore et al., 1990; Vargas et al., 1991), and rat aorta is particularly sensitive to the inhibitory effect of N^G-nitro-L-arginine on the relaxation response (Vargas et al., 1991). The second reason is that acetylcholine may stimulate the release of EDRF from a source distinct from L-arginine, which may cause the resistant-relaxation response. However, this explanation is also unlikely to apply, because the resistant-relaxation response to acetylcholine was not inhibited by oxyhemoglobin which suppresses the endothelium-dependent relaxation by binding EDRF (Martin et al., 1985), and because the increase in cGMP induced by acetylcholine was completely inhibited by N^G-nitro-L-arginine. Furthermore, as the resistant relaxation was not affected by indomethacin, the relaxation response is not due to cyclooxygenase products such as prostacyclin. The resistant relaxation is also not due to the low concentration of norepinephrine (5×10^{-9} M) used to produce precontraction, because papaverine caused comparable relaxations in the vascular strips precontracted with 5×10^{-9} M norepinephrine in the presence of N^G-nitro-L-arginine and with 10^{-7} M norepinephrine in its absence, and because the precontractile levels caused by norepinephrine in the two experimental conditions were similar. In addition, tetraethylammonium or KCl did not affect papaverine-induced relaxation, which suggests that their inhibitory effect on the resistant relaxation is not due to a non-specific action. Thus, the acetylcholine-induced relaxation resistant to N^G-nitro-L-arginine is most likely to have been evoked by the release of relaxing factor(s) other than EDRF or prostacyclin.

Acetylcholine-induced hyperpolarization in rat aorta is due to the opening of membrane K^+ channels (Chen et al., 1988; Taylor et al., 1988). The amplitude of the hyperpolarization is increased in low-K^+ solutions and decreased in high-K^+ solutions. In rat arteries, the extracellular K^+ solution expected to block the acetylcholine-induced hyperpolarization was 20–25 mM (Chen and Suzuki, 1989). In the present study, acetylcholine did not cause relaxation in the vascular strips precontracted with 20 mM KCl in the presence of N^G-nitro-L-arginine. Furthermore, tetraethylammonium, a K^+ channel blocker with broad specificity, abolished the N^G-nitro-L-arginine-resistant relaxation. Although antagonistic actions of tetraethylammonium on muscarinic receptors have been reported in the rat central nervous system (Balduini et al., 1990), the inhibitory effect of tetraethylammonium on the relaxation response is not due to blockade of the acetylcholine receptors, because acetylcholine produced a mean maximal relaxation of 52.4% in the solution containing tetraethylammonium but not N^G-nitro-L-arginine. On the other hand, glibenclamide, an ATP-sensitive K^+ channel blocker, did not inhibit the relaxation response to acetylcholine resistant to N^G-nitro-L-arginine. This result agrees with that of a previous study showing that, in contrast to tetraethylammonium, glibenclamide did not inhibit acetylcholine-induced $^{42}K^+/^{86}Rb^+$ efflux (Bray and Quast, 1991) or hyperpolarization (Fujii et al., 1992) in rat aorta, suggesting that the hyperpolarization is not mediated by the ATP-sensitive K^+ channel. Thus, the resistant relaxation response to acetylcholine is most likely to be mediated by EDHF. Also, acetylcholine-induced relaxation in the presence of tetraethylammonium was further reduced by 10 mM KCl. The further inhibitory effect of KCl could not have been due to the lack of tetraethylammonium blockage of K^+ channels, because the relaxation response to acetylcholine resistant to N^G-nitro-L-arginine was completely blocked by tetraethylammonium. One possible explanation is that KCl may inhibit cGMP-mediated relaxation as well as cGMP-independent relaxation (i.e., EDHF), because the relaxation response to sodium nitroprusside which releases nitric oxide may be smaller in the vascular strips precontracted with KCl than with norepinephrine (Taylor et al., 1988).

In the present study, the N^G-nitro-L-arginine-resistant relaxation response to acetylcholine was unmasked when the precontraction was produced by a low concentration of 5×10^{-9} M norepinephrine. One possible explanation for this is that the relaxation mediated by EDHF may be related to the degree of depolarization of the vessel induced by norepinephrine. As depolarization of smooth muscle cells may

become smaller with lower concentrations of norepinephrine, the effect of hyperpolarization on the relaxation is considered to become larger. However, acetylcholine induced a significantly greater hyperpolarization in the presence of norepinephrine (i.e., depolarization occurred) than in its absence (Bény and Brunet, 1988b; Chen et al., 1988; Keef et al., 1992). Thus, the reasons for the unmasking of the N^G-nitro-L-arginine-resistant relaxation with a lower concentration of norepinephrine remain unknown. In rat mesenteric arteries, acetylcholine caused a maximal relaxation of more than 60% even in the vascular rings precontracted with a high concentration of 10^{-5} M norepinephrine in the presence of 3×10^{-5} M N^G-nitro-L-arginine (Fujii et al., 1992). In the present study, the N^G-nitro-L-arginine-resistant relaxation appeared only with use of a low concentration of norepinephrine. It also seems likely that, in rat aorta, receptors related to the release of EDHF are less efficiently coupled to the agonist than are the receptors for the release of EDRF. Thus, EDHF seems to play a minor role in the relaxation response to acetylcholine in rat aorta but may make a larger contribution to the relaxation response in the small or resistance vessels than in the conduit vessels such as those of the aorta.

In conclusion, in rat aorta, the relaxation in response to acetylcholine which is resistant to N^G-nitro-L-arginine appeared only when lower concentrations of the precontractile agent, norepinephrine, were used. This N^G-nitro-L-arginine-resistant relaxation is mediated by a cGMP-independent mechanism, possibly EDHF, and appears not to play a major role in the relaxation response to acetylcholine in the absence of N^G-nitro-L-arginine.

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