Stimulation of Soluble Guanylate Cyclase by an Acetylcholine-Induced Endothelium-Derived Factor from Rabbit and Canine Arteries

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SUMMARY. The present study was designed to investigate the hypothesis that, during acetylcholine-induced endothelium-dependent relaxation, a factor(s) is released from endothelial cells which directly activates soluble guanylate cyclase. We attempted to determine what similarities or differences existed between this factor and endothelium-derived relaxing factor. The study was performed on segments of rabbit aorta and canine femoral artery. Purified soluble guanylate cyclase was injected into the lumen of these vascular segments, together with its substrate, for intraluminal incubation of the enzyme. In endothelium-intact vascular segments, the activity of guanylate cyclase was enhanced over control values obtained by incubation in test tubes. The stimulation was further increased by acetylcholine in concentrations which caused relaxation of the vascular segments. The stimulating principle could not be transferred from the vessel lumen to an external solution of guanylate cyclase, indicating a short life-time. Removal of the endothelium prevented formation and release of the guanylate cyclase stimulating factor(s). Atropine, mepacrine, or nordihydroguaiaretic acid, which inhibit acetylcholine-induced endothelium-dependent relaxations, also inhibited acetylcholine-induced endothelium-mediated activation of guanylate cyclase. The results support the hypothesis that acetylcholine-induced endothelium-derived relaxing factor increases cyclic guanosine monophosphate levels of vascular smooth muscle by a stimulation of soluble guanylate cyclase. (Circ Res 58: 531-538, 1986)
sected out. Mongrel dogs (25–35 kg) were anesthetized with sodium pentobarbital, and the femoral artery and larger side branches were excised. Arterial segments of a length of 3 cm (rabbit aorta and canine femoral artery) or 1 cm (rabbit femoral arteries and side branches of canine femoral arteries) were prepared free of connective tissue. Any damage of the endothelium was avoided during the preparation of the vascular segments. In some experiments, the endothelium was removed by gently rubbing the intimal surface with a rough steel cannula or by inverting the segments and carefully removing the endothelium with a razor blade. Both procedures proved to be equieffective as indicated by the complete failure of ACh to induce relaxation and confirmed by light microscopy after silver staining (Poole et al., 1958). Contractile responses to norepinephrine (NE) were not affected by either rubbing procedure.

After ligation of the side branches, each segment was cannulated with two L-shaped stainless steel cannulas and fixed with ligatures. The segment was stretched to its approximate in situ length, and the vertical parts of the cannulas were fixed in a plastic frame at this distance. This device containing the segment was then placed in a special organ bath so that the top ends of the cannulas protruded beyond the surface (cf. Busse et al., 1983, 1984). The bath contained oxygenated Tyrode’s solution (PO2 ≥ 460 mm Hg, pH 7.4, 37°C) as the extraluminal medium. The proximal end of the inflow cannula was connected to a perfusion pump and the segment was perfused (0.66 ml/min) with modified Tyrode’s solution containing NE (0.1 µM), glutathione (0.5 mM), MgCl2 (3 mM), and bovine γ-globulin (0.1 mg/ml; PO2 ≈ 140 mm Hg). The pH of the solution was adjusted to 7.4 at 37°C by gassing with 5% CO2/20% O2 and the segment was perfused (0.66 ml/min) by gassing with 5% CO2/20% O2, and the segment was perfused (0.66 ml/min) with modified Tyrode’s solution containing NE (0.1 µM), glutathione (0.5 mM), MgCl2 (3 mM), and bovine γ-globulin (0.1 mg/ml; PO2 ≈ 140 mm Hg). The pH of the solution was adjusted to 7.4 at 37°C by gassing with 5% CO2/20% O2 and 75% N2. Glutathione and bovine γ-globulin are essential for the determination of the soluble GC activity described below. Glutathione protects soluble GC from oxidative inactivation. Bovine γ-globulin prevents adsorption of the purified GC to different surfaces, e.g., test tubes.

To assess mechanical responsiveness, vascular segments were perfused with modified Tyrode’s solution containing NE (0.1 µM) until a contraction plateau had been reached. Then ACh (0.01–2 µM) was added to induce relaxation. To test the effects of drugs in modified Tyrode’s solution, vascular segments were perfused for 60 minutes with mepacrine (30 µM), norethidroguaiaretic acid (20 µM), or indomethacin (20 µM), respectively, before exposure to NE and ACh. In some experiments sodium nitroprusside (SNP, 1 nM to 1 µM) was added instead of ACh.

Measurement of Smooth Muscle Tone

The experiments were performed with endothelium-intact rabbit aortas and canine femoral arteries as the “donor” vessels for EDRF. For bioassay of EDRF released into its lumen, the “donor” vessel was perfused in series with endothelium-denuded “detector” vessel (rabbit femoral artery or side branch of canine femoral artery) as described by Forstermann et al. (1984). The tone of the vascular segments was measured by continuously recording the external diameters at their midpoints using photodensitometric devices (Busse et al., 1983, 1984). For this bioassay of EDRF, the mechanical responses of “donor” and “detector” segments were recorded simultaneously.

Purification of Soluble Guanylate Cyclase from Bovine Lung

Soluble, heme-containing GC was purified from bovine lung at apparent homogeneity according to the method described by Gerzer et al. (1981), but with some modifications. We used Sepharose CL-6B containing 1 µmol Cibacron blue F3G-A/ml swollen gel prepared according to Heyns and De Moor (1974). It allows elution of GC under low ionic strength conditions with a yield of about 50%. The properties of the isolated enzyme with an approximate molecular weight of 150,000 daltons were described briefly (Mülsch and Böhme, 1984).

Determination of the Activity of Soluble Guanylate Cyclase

The incubation procedure to determine the activity of purified soluble GC was carried out in test tubes as well as in the lumen of vascular segments. Purified soluble GC (10–100 nm), with and without drugs added, was preincubated in test tubes for 3 minutes at 37°C and pH 7.4 in modified Tyrode’s solution containing 5% CO2, 20% O2, and 75% N2, or in a triethanolamine/HCl-buffer (50 mM) containing glutathione (0.5 mM), MgCl2 (3 mM), and bovine γ-globulin (0.1 mg/ml). All concentrations given are final concentrations at the respective step. The incubations were started by addition of [α-32P]GTP (0.1 mM, 0.2 µCi). If the effect of SNP on the activity of GC was to be evaluated, SNP was added together with [α-32P]GTP. After 3 minutes, the reactions were stopped by addition of zinc acetate (50 mM) and subsequently sodium carbonate (55 mM). Isolation of cGMP and calculation of guanylate cyclase activity were performed as described (Schultz and Böhme, 1984). Control experiments for the determination of non-enzymatically formed gMP were performed in the absence of guanylate cyclase. For the determination of recovery of GMP during the incubation procedure, cyclic [3H]GMP (0.05 mM, 20 nCi) was used.

Incubation in Test Tubes

Initially, we investigated whether the effluent of endothelium-intact rabbit aortic segments stimulates purified soluble GC in a test tube. Modified Tyrode’s solution containing NE (0.1 µM) with and without ACh (2 µM) was injected into the lumen of rabbit aorta segments with intact endothelium, preincubated for 0.5 and 3 minutes, and transferred within 5 or 60 seconds to test tubes containing purified soluble GC. Subsequently, the incubations were started by addition of [α-32P]GTP (0.1 mM, 0.5 µCi). The reactions were stopped after 25 or 180 seconds, as described above. In control experiments preincubations of NE with and without ACh were performed in test tubes instead of vascular segments.

Incubations of Guanylate Cyclase Injected into the Lumen of Vascular Segments

To circumvent technical difficulties arising from the short half life of ACh-induced EDRF, we determined the activity of purified soluble GC after injecting it into the lumen of vascular segments. The segment was at first perfused and tested for mechanical responsiveness to ACh, as described above. Then the perfusion was stopped.
and the perfusion system was disconnected from the top ends of the L-shaped steel cannulas while the arterial segment remained in place in the organ bath. The remaining intraluminal medium was cleared from the segment by quickly removing the device containing the arterial segment from the organ bath, turning it sideways, and passing a gentle stream of air through the lumen. The segment was promptly returned into the organ bath. Then, a preincubated (3 minutes, 37°C) purified soluble GC (34–170 nM) in modified Tyrode’s solution was mixed with [α-32P]GTP (0.1 mM, 0.5 μCi) and cyclic [3H]GMP (0.05 mM, 20 nCi) and injected within 20 seconds into the lumen of the vascular segments. The mixture contained cyclic [3H]GMP to determine the recovery of cyclic GMP. The ends of both cannulas were closed with plugs and the incubation was allowed to proceed for a specified time. The cannulas were then reopened, and the intraluminal incubation mixture was rapidly transferred from the vascular lumen into the zinc acetate solution by gently blowing air through the segment as described above. Following this, the vascular segment was reconnected to the perfusion system. The isolation and determination of the cyclic [32P]GMP formed and calculation of GC activity was performed as described (Schultze and Böhme, 1984). Control experiments were performed in test tubes instead of vascular segments under comparable conditions. Blank values were obtained by omitting purified soluble GC from the reaction mixture and were the same in test tubes and isolated vascular segments.

After the first intraluminal determination of soluble GC activity, the vascular segment was perfused for at least 16 minutes to wash out residual GC-containing solution prior to the next incubation procedure. A maximum of five runs was performed on each segment. In the experiments in which the effects of drugs on EDRF formation and/or release were to be evaluated, the activity of GC injected into the lumen of vascular segments was first determined in the presence of NE (0.1 μM) and then in the presence of both NE (0.1 μM) and ACh (2 μM). Subsequently, the segments were perfused for 60 minutes with modified Tyrode’s solution containing the drug under study. Then, the determinations of GC activity were repeated in the presence or absence of the drug.

GC activities are expressed as percent of test tube controls (mean ± SE). Differences were tested for significance by Student’s t-test. Significance was accepted at the 0.05 level of probability.

Materials
Norepinephrine·HCl, acetylcholine·HCl, mecaprine·HCl, nordihydroguaiaretic acid, indomethacin, and glutathione were purchased from Sigma. Atropine sulfate was obtained from Drobena, and bovine γ-globulin was from Serva. Other materials for the purification of soluble GC and determination of enzyme activity were obtained as previously described (Gerzer et al., 1981). Stock solutions of drugs were prepared immediately before use in different solvents, and were then diluted with modified Tyrode’s solution or triethanolamine/HCl buffer. Nordihydroguaiaretic acid was dissolved in modified Tyrode’s solution (37°C) and indomethacin in 25% (vol/vol) ethanol, 0.675% (wt/vol) NaCl, and 0.75% (wt/vol) NaHCO3; all other drugs were dissolved in twice-distilled water.

Results

Relaxation of Vascular Segments by Acetylcholine and Sodium Nitroprusside
ACh (0.01–2 μM) induced relaxations of precontracted (NE, 0.1 μM), endothelium-intact rabbit aortas, and canine femoral arteries in modified Tyrode’s solution. Similarly, the ACh-induced release of EDRF into the lumen of endothelium-intact “donor” rabbit aortic segments and the transfer of this EDRF to endothelium-denuded “detector” rabbit femoral segments could be demonstrated in modified Tyrode’s solution (Fig. 1). GTP (0.1 mM) and cGMP (0.05 mM) had no effect on ACh-induced relaxation and intraluminal release of EDRF (data not shown). Furthermore, ACh-induced relaxations of “donor” as well as “detector” vascular segments were abolished in the presence of atropine (2 μM), after pretreatment of the “donor” segments for 60 minutes with mecaprine (30 μM) or nordihydroguaiaretic acid (20 μM), and after removal of the endothelium from the “donor” segments. Thus, the arteries showed identical mechanical response in modified as in unmodified Tyrode’s solution (cf. Förstermann et al., 1984).

Figure 1. Representative diameter recordings (D) of an endothelium-intact rabbit aortic segment (“donor” segment, upper panel) and of an endothelium-denuded rabbit femoral segment (“detector” segment, lower panel) perfused in series with modified Tyrode’s solution. The effluent from the endothelium-intact vascular segment was subsequently perfused through the endothelium-denuded segment as described previously (Förstermann et al., 1984). Both vascular segments were precontracted by perfusion with norepinephrine (NE, 0.1 μM). Addition of acetylcholine (ACh, 0.05 and 2 μM) induced relaxation of the endothelium-intact vascular segment and the release of EDRF from the “donor” segment which relaxed the endothelium-denuded “detector” segment. The constituents of modified Tyrode’s solution, glutathione (0.5 mM), MgCl2 (3 mM), and bovine γ-globulin (0.1 mg/ml), as well as GTP (0.1 mM) and cGMP (0.05 mM), did not affect ACh-induced formation and/or release of EDRF.
The NO containing vasodilator SNP in a concentration of 10 nM caused the same degree of relaxation of precontracted (NE, 0.1 μM) endothelium-free rabbit femoral arteries as did the effluent of endothelium-intact arteries after stimulation with ACh (2 μM).

**Activity of Purified Soluble Guanylate Cyclase**

The activity of soluble, heme-containing GC purified from bovine lung was significantly inhibited with modified Tyrode's solution instead of the triethanolamine/HCl buffer described in methods (Table 1). Basal GC activity determined in modified Tyrode's solution was increased about 2-fold by omission of NaCl (134 mM) or CaCl₂ (1.6 mM), respectively. In the absence of NaCl and CaCl₂, the enzyme activity was comparable to that determined in triethanolamine/HCl buffer. Correspondingly, GC activity determined in triethanolamine/HCl buffer was reduced to about 30% by the addition of NaCl or CaCl₂. The enzyme activity in the presence of NaCl and CaCl₂ was comparable to that measured in modified Tyrode's solution (Table 1).

Also, the stimulation of soluble GC by SNP was reduced in modified Tyrode's solution. Maximal stimulation achieved with SNP was about 60-fold in triethanolamine/HCl buffer, but only about 20-fold in modified Tyrode's solution, both at about 20 μM SNP. The SNP concentration for half-maximum stimulation was about 2 μM, independent of the buffer used (data not shown). At 1 μM SNP the extent of stimulation was about 20-fold in triethanolamine/HCl buffer and about 8-fold in modified Tyrode's solution (Table 1).

<table>
<thead>
<tr>
<th>Buffer system</th>
<th>Guanylate cyclase activity (nmol/mg protein per min)</th>
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<tbody>
<tr>
<td></td>
<td>Without SNP</td>
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<tr>
<td>Triethanolamine/HCl</td>
<td>67.0 ± 10.0</td>
</tr>
<tr>
<td>+ CaCl₂</td>
<td>25 ± 4.7</td>
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<tr>
<td>+ NaCl</td>
<td>20 ± 4.2</td>
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<tr>
<td>+ CaCl₂ + NaCl</td>
<td>9 ± 2.4</td>
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<tr>
<td>Tyrode's solution</td>
<td>10.0 ± 2.2</td>
</tr>
<tr>
<td>- CaCl₂</td>
<td>20 ± 3.0</td>
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<tr>
<td>- NaCl</td>
<td>24 ± 2.8</td>
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<tr>
<td>- CaCl₂ - NaCl</td>
<td>68.3 ± 6.5</td>
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The activity of soluble, heme-containing guanylate cyclase purified from bovine lung was determined in the presence and absence of sodium nitroprusside (SNP, 1 μM). Incubations were carried out in triethanolamine/HCl (5 mM) buffer or modified Tyrode's solution, as described in Methods. CaCl₂ (1.6 mM) and NaCl (134 mM) were added (+) or omitted (−) from the incubation medium. Data given are mean ± s.e of three experiments performed in triplicates.

**Inhibition of ACh-Induced Stimulation of Purified Soluble Guanylate Cyclase**

The activity of purified soluble GC injected into the lumen of endothelium-denuded rabbit aortic

<table>
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segments was not significantly different from test tube controls (Fig. 4a). In addition, the ACh-induced stimulation of GC was abolished in these segments (Fig. 4a). The addition of atropine (2 μM) significantly reduced ACh-induced stimulation of GC in endothelium-intact segments to 163 ± 20% (n = 8) of control values, whereas basal (ACh-independent) enzyme stimulation due to endothelial cells was not significantly altered (Fig. 4b). Pretreatment of endothelium-intact rabbit aortic segments for 60 minutes with mepacrine (30 μM) or nordihydroguaiaretic acid (20 μM) significantly reduced ACh-induced stimulation of GC to 198 ± 17% (n = 6) or 221 ± 10% (n = 6) of control values, respectively (Fig. 4, c and d). These effects are independent of the presence of mepacrine or nordihydroguaiaretic acid, during the intraluminal incubation of soluble GC (data not shown). Pretreatment of the vascular segments with indomethacin (20 μM) did not significantly alter ACh-induced GC stimulation (Fig. 4e). The ACh-independent and basal endothelium-dependent stimulation of GC was not affected by these drugs (Fig. 4, c-e).

Discussion

To demonstrate the proposed stimulatory effect of an ACh-induced endothelial factor on soluble GC, it was necessary to use a medium which does not disturb the integrity and responsiveness of the endothelial cell membrane with respect to the formation and release of endothelial relaxing factor(s). On the other hand, this medium must allow the determination of the activity of a purified soluble GC. To meet the first demand, we had to use an extracellular medium like Tyrode's solution; however, additions such as glutathione, γ-globulin, GTP, cGMP, and additional MgCl₂ were necessary to achieve a sufficient GC activity and recovery of the cGMP formed. The constituents of Tyrode's solution NaCl and CaCl₂ reduced significantly the activity of the purified soluble, heme-containing GC, and, to a greater degree, the enzyme stimulation by SNP. The effect of NaCl is probably caused by the increased ionic strength of the buffer. Effects of Ca²⁺ on soluble GC have been described by several
authors. So far, these studies show conflicting results, probably because different enzyme preparations were used. Inhibitory effects of Ca++ on the stimulation of soluble GC by NO-containing compounds but not on the basal enzyme activity have been described in more detail, e.g., by Gruetter et al. (1980). The effect of Ca++ as well as ionic strength on the purified, soluble, heme-containing guanylate cyclase certainly deserves further investigation.

If purified soluble GC was injected, together with its substrate, into the lumen of endothelium-intact rabbit aortic or canine femoral arterial segments, a significant stimulation of the enzyme was observed in the absence of ACh. Since removal of endothelium reduced the enzyme activities to values obtained in the test tubes, this points to a basal release of a GC-stimulating factor(s) from endothelial cells. Similarly, a basal release of ERDF has recently been demonstrated by Griffith et al. (1984). Furthermore, it has been shown that removal of endothelium decreases cGMP levels in the subjacent smooth muscle cells (Holzmann, 1982; Diamond and Chu, 1983; Rapoport and Murad, 1983a, 1983b; Furchgott et al., 1984; Miller et al., 1984).

The activity of the purified soluble GC injected into the lumen of vascular segments was further stimulated by ACh in a concentration-dependent manner. This stimulation was prevented by removal of endothelium or by the addition of atropine. These results indicate that ACh-induced stimulation of soluble GC is endothelium dependent and is mediated by muscarinic receptors. The same has been shown for ACh-induced formation and/or release of EDRF (Furchgott and Zawadzki, 1980).

The extent of the ACh-induced stimulation of soluble GC was independent of the incubation time. The stimulatory factor(s) obviously reached a steady state level, suggesting a short life time as described for the ACh-induced EDRF. Similarly, we were unable to demonstrate any significant stimulation of soluble GC in test tubes by the effluent of endothelium-intact rabbit aortic segments treated with ACh. This further indicates that the stimulating activity does not accumulate in the vascular lumen, and is obviously chemically unstable, as has been reported for EDRF (Griffith et al., 1984; Förstermann et al., 1984).

ACh-induced stimulation of soluble GC was inhibited after pretreatment of endothelium-intact rabbit aortic segments with mepacrine or nordihydroguaiaretic acid at concentrations that also inhibited the ACh-induced formation and/or release of EDRF (Furchgott and Zawadzki, 1980; Chand and Altura, 1981; Furchgott, 1983, 1984; Förstermann et al., 1984; Förstermann and Neufang, 1984). The blockade of the ACh-induced GC stimulation was
evident, even though these drugs were absent during the actual incubation. Thus, inhibition takes place at the level of the endothelium, and is not caused by an interaction of these drugs with soluble GC itself. The ineffectiveness of indomethacin to inhibit ACh-induced stimulation of soluble GC implies that the formation and/or release of the stimulating principle is insensitive to indomethacin, at least in the concentration used, as shown for EDRF (Furchgott and Zawadzki, 1980; Furchgott, 1983, 1984; Förstermann and Neufang, 1984).

It has previously been reported that platelet membranes can release fatty acids that modulate GC activity (Gerzer et al., 1983). Arachidonic acid or linoleic acid are known to stimulate basal activity of soluble GC (Böhme et al., 1983; Gerzer et al., 1983; Wolin and Ignarro, 1983). However, it is unlikely that these fatty acids, or any of their relatively stable metabolites, are responsible for the activation of GC observed here, since the GC stimulating factor(s) obviously has a short life time.

The extent of ACh-induced increase in GC activity shown here would not account for the increase of cGMP levels in rabbit aorta (Diamond and Chu, 1983; Furchgott and Jothianandan, 1983; Furchgott et al., 1984). However, the intracellular medium is different from the intraluminal medium in which the determination of soluble GC was performed. The basal enzyme activity, and especially the stimulation by SNP, were reduced in modified Tyrode’s solution. Therefore, it is likely that the effect of the ACh-induced stimulatory factor(s) on GC is reduced as well. Furthermore, the release of ACh-induced EDRF seems to be greater in the abluminal than in the intraluminal direction (Busse et al., 1985).

In conclusion, the results presented demonstrate that endothelial cells, of at least two types of arteries (rabbit aorta and canine femoral artery), produce a labile factor(s) which stimulates soluble GC. The formation and release of this factor is increased by ACh. The factor may be identical with the ACh-induced EDRF. This assumption is supported by correlations between known properties of EDRF and those found for the GC-stimulating factor(s). Thus, stimulation of GC, at least of the soluble enzyme form, could be responsible for the increase in cGMP levels observed in vascular smooth muscle cells during endothelium-dependent relaxation.

We would like to acknowledge the excellent technical assistance of Cornelia Kellner.

This work was presented in part at the Spring Meeting of the German Pharmacological Society, March 1985 (Mulsch et al., 1985).

The work was supported by the Deutsche Forschungsgemeinschaft (Grants Schu 2/14-5 and Bu 436/2-2).

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Received December 11, 1984; accepted for publication October 3, 1985.

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INDEX TERMS: Endothelium-derived relaxing factor • Soluble guanylate cyclase • Acetylcholine • Atropine • Mepacrine •
Nordihydroguaiaretic acid
Stimulation of soluble guanylate cyclase by an acetylcholine-induced endothelium-derived factor from rabbit and canine arteries.
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Circ Res. 1986;58:531-538
doi: 10.1161/01.RES.58.4.531

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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http://circres.ahajournals.org/content/58/4/531