SUMMARY. The present study investigates the hypothesis that endothelium-dependent relaxation of vascular smooth muscle may be mediated through the formation of cGMP. Relaxation of the rat thoracic aorta to acetylcholine, histamine, and Ca** ionophore A23187 was associated with increased levels of cGMP in a time- and concentration-dependent manner, whereas cAMP levels were unaltered. Removal of the endothelium prevented relaxation to these agents and prevented the increased levels of cGMP. Removal of the endothelium after exposure to acetylcholine only partially decreased the elevated levels of cGMP, suggesting that the changes in cGMP occurred within the smooth muscle cells. Eicosatetraynoic acid, an inhibitor of lipoygenase and cyclooxygenase, and quinacrine, an inhibitor of phospholipase, prevented and reversed acetylcholine-induced relaxation, respectively, and inhibited acetylcholine-induced increased levels of cGMP. In contrast, sodium nitroprusside-induced relaxation and increased levels of cGMP were independent of the presence of the endothelium, exposure to eicosatetraynoic acid, and quinacrine. The present results support the hypothesis that vascular smooth muscle relaxation induced by some agents is dependent on the presence of the endothelium and is mediated through the formation of an endothelial factor that increases cGMP levels in smooth muscle. (Circ Res 52: 352-357, 1983)

RELAXATION of numerous vascular smooth muscle preparations by various pharmacological agents has been shown to be dependent upon the presence of the endothelium (Furchgott and Zawadski, 1980; Lee, 1980; Chand and Altura, 1981; Altura and Chand, 1981; De Mey and Vanhoutte, 1981; Furchgott, 1981; Van de Voorde and Leusen, 1982; Lee, 1982; Singer and Peach, 1982). However, the mechanism by which the endothelium leads to relaxation of the smooth muscle is not known. It has been proposed (Furchgott and Zawadski, 1980) that exposure of the endothelial cells to various agents induces the release of a substance which, following a series of events, relaxes the smooth muscle. The substance released is thought to be arachidonic acid or some other unsaturated fatty acid, since quinacrine, an inhibitor of phospholipase A2, inhibits the endothelium-dependent agonist-induced relaxation (Furchgott and Zawadski, 1980). It has also been hypothesized that the fatty acid may be oxidized, since anoxia, 5,8,11,14-eicosatetraynoic acid (ETYA) and nordihydroguaiaretic acid, inhibitors of cyclooxygenase and/or lipoygenase, inhibited the relaxation (Furchgott and Zawadski, 1980; Furchgott, 1981). The oxidized product may be a labile hydroperoxide or free radical, since hydroquinone, an antioxidant and free radical scavenger, inhibited the relaxation.

The effects of nitroprusside, nitroglycerin, and other nitro-vasodilators on smooth muscle relaxation are thought to be mediated through the activation of guanylate cyclase and cGMP formation (Katsuki and Murad, 1977; Katsuki et al., 1977; Schultz et al., 1977). These agents probably lead to the formation of nitric oxide which activates guanylate cyclase (Katsuki et al., 1977; Arnold et al., 1977). Guanylate cyclase can also be activated with hydroxyl free radical (Mittal and Murad, 1977), unsaturated fatty acids (Walach and Pastan, 1976; Glass et al., 1977), and lipid peroxides (Hidaka and Asano, 1977).

The purpose of our study was to test the hypothesis that relaxation of vascular smooth muscle with other agents, which are dependent upon the endothelium, may also be mediated through the formation of cGMP within the smooth muscle. The effect of acetylcholine, histamine, A23187, and sodium nitroprusside on relaxation and the levels of cGMP and cAMP in the presence and absence of endothelium was investigated. Furthermore, the effects of quinacrine and ETYA on relaxation and cGMP levels induced by acetylcholine and nitroprusside were investigated. Some of these studies have been reported in abstract form (Rapoport et al., 1983).

Methods

Relaxation Studies

Rats (Sprague-Dawley, male, 200–300 g) were decapitated, and their thoracic aortas were removed and cut into spiral strips (approximately 2 mm × 1 cm). Strips were mounted in organ baths containing Krebs-Ringer bicarbonate solution which was gassed with 95% O2-5% CO2 and had the following composition (mM): NaCl, 118.5; KCl, 4.74; MgSO4, 1.18; KH2PO4, 1.18; CaCl2, 2.5; NaHCO3, 24.9; glucose, 10. Resting tension of 0.4 g - force was maintained.
throughout the experiment. Tissues were allowed to equilibrate for 2 hours prior to the addition of any drugs.

Responses to 0.1 μM norepinephrine then were elicited, followed by the addition of acetylcholine, histamine, A23187, or sodium nitroprusside. The intimal surface of some of the strips then was rubbed gently with a scalpel, which removed the endothelial layer and left the internal elastic lamina intact, as confirmed by means of scanning electron microscopy. Following reequilibration (30–60 min), subsequent contractile responses to 0.1 μM norepinephrine were unaffected by the rubbing procedure. Acetylcholine, histamine, A23187, or nitroprusside then was added. Other strips were pretreated with ETYA (0.1 mM) for 30 minutes, followed by the addition of norepinephrine and the smooth muscle relaxants. Still other tissues were exposed to quinacrine (0.1 mM) for 30 seconds after relaxations to acetylcholine, histamine, A23187, and nitroprusside had been elicited.

cGMP and cAMP Measurements
Rubbed or unrubbed spiral strips were prepared as described above and were allowed to equilibrate in flasks containing Krebs-Ringer bicarbonate solution gassed with 95% O₂–5% CO₂. Various times after exposure to the agents indicated, the tissues were frozen in liquid nitrogen, and cyclic nucleotide levels were assayed as previously described (Katsuki and Murad, 1977). Briefly, frozen tissues were homogenized in 6% trichloroacetic acid and samples were centrifuged. Supernatant fractions were extracted with ether and radioimmunoassayed for cyclic nucleotides (Steiner et al., 1972; Kimura et al., 1974). Similar results were obtained when tissues were mounted in organ baths and exposed to the agents indicated (Rapoport and Murad, unpublished observation). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Significance was accepted at the 0.05 level of probability, using Student’s t-test unless otherwise indicated.

Materials
Acetylcholine-HCl, 1-norepinephrine-HCl, histamine-HCl, sodium nitroprusside, and quinacrine-HCl were obtained from Sigma, 6S-[6α(2S*,3S*), 8β(R*), 9β, 11α]-5-methyl-4-benzoxazolecarboxylic acid (A23187) from Calbiochem and 5,8,11,14-eicosatetraynoic acid (ETYA) was a gift from Hoffman-La Roche. Other materials were obtained as previously described (Kimura et al., 1974; Kimura et al., 1975; Mittal and Murad, 1977).

Results

Relaxation Studies
The results of the relaxation studies are similar to those of other investigators (Furchgott and Zawadski, 1980; Furchgott, 1981; Singer and Peach, 1982; Van de Voorde and Leusen, 1982) and, thus, most of the relaxation data are not presented. The time course for relaxation induced by maximally effective concentrations of acetylcholine, histamine, and A23187 is shown in Figures 1, 2, and 3, respectively. Relaxation to acetylcholine and histamine occurred within 5 seconds of their addition, whereas relaxation to A23187 occurred within 10 seconds of addition. Neither relaxation nor contraction to these agents was observed in tissues whose endothelium had been removed and then were contracted with 0.1 μM norepinephrine (data not shown). In contrast, relaxation induced by a maximally effective concentration of nitroprusside (1 μM) completely relaxed both rubbed and control tissues (Table 1). Maximal relaxation occurred within 1.5 minutes of addition of nitroprusside.

Pretreatment with ETYA (0.1 mM) for 30 minutes prevented relaxation induced by maximally effective concentrations of acetylcholine, histamine, and A23187 (Table 2; some data not shown). However, relaxation induced by 1 μM nitroprusside was unaffected by ETYA (Table 2). Pretreatment with ETYA (0.1 mM) decreased the contractile response to nor-

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cGMP (pmol/mg protein)</th>
<th>Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.2 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>Rubbed</td>
<td>0.5 ± 0.2*</td>
<td>0</td>
</tr>
<tr>
<td>Nitroprusside (1 μM)</td>
<td>66.5 ± 12.1†</td>
<td>100</td>
</tr>
<tr>
<td>Rubbed + nitroprusside (1 μM)</td>
<td>82.6 ± 18.1†</td>
<td>100</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± se; n = 7 in each case. Aortas were rubbed (see Methods) or allowed to remain intact. Tissues then were exposed to 0.1 μM norepinephrine for 5 minutes, followed by 1 μM nitroprusside for 1.5 minutes, frozen, and assayed for cGMP (see Methods).

* Significantly less than control.
† Significantly greater than control.

### Table 2

<table>
<thead>
<tr>
<th>Addition</th>
<th>cGMP (pmol/mg protein)</th>
<th>Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.8 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>ETYA (0.1 mM)</td>
<td>0.5 ± 0.1*</td>
<td>0</td>
</tr>
<tr>
<td>Acetylcholine (10 μM)</td>
<td>11.3 ± 2.5†</td>
<td>85.6 ± 3.8</td>
</tr>
<tr>
<td>ETYA (0.1 mM) + acetylcholine (10 μM)</td>
<td>0.8 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2.4 ± 1.7</td>
<td>0</td>
</tr>
<tr>
<td>ETYA (0.1 mM)</td>
<td>0.4 ± 0.1*</td>
<td>0</td>
</tr>
<tr>
<td>Nitroprusside (1 μM)</td>
<td>52.6 ± 3.5†</td>
<td>100</td>
</tr>
<tr>
<td>ETYA (0.1 mM) + nitroprusside (1 μM)</td>
<td>29.9 ± 6.0‡</td>
<td>100</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± se; n = 4 in each experiment. Tissues were exposed to 0.1 mM ETYA for 30 minutes followed by 0.1 μM norepinephrine for 5 minutes. Other tissues remained unexposed to ETYA. Acetylcholine (10 μM) or nitroprusside (1 μM) was then added for 1 and 1.5 minutes, respectively. Tissues then were frozen and assayed for cGMP (see Methods).

* Significantly less than control.
† Significantly greater than control.
‡ Significantly less than nitroprusside-treated tissues.
epinephrine (0.1 μM) by 18.6 ± 4.5% (mean ± se; P < 0.05, paired t-test; n = 9 in each case). ETYA (0.1 mM) added following the relaxation caused only a small reversal.

Relaxation induced by maximally effective concentrations of acetylcholine and histamine was partially reversed by quinacrine (36% and 30%, respectively; Table 3). In contrast, relaxations induced by 0.3 μM A23187 and 1 μM nitroprusside were not reversed by 0.1 mM quinacrine. Quinacrine (0.1 mM) itself caused complete relaxation of strips contracted with 0.1 μM norepinephrine within approximately 8 minutes of its addition.

**Effect of Acetylcholine, Histamine, A23187, and Nitroprusside on Cyclic Nucleotide Levels**

The increase in levels of cGMP induced by acetylcholine (10 μM), histamine (1 mM) and A23187 (0.3 μM) was abolished in tissues that were rubbed (Figs. 1–3). When endothelium was present, significant increases in cGMP occurred within 5 seconds of addition of acetylcholine (10 μM) and histamine (1 mM) and within 10 seconds of addition of A23187 (0.3 μM). cAMP levels were unaltered by exposure to acetylcholine (Fig. 1), histamine, or A23187 (data not shown). Basal levels of cGMP and cAMP were decreased in those tissues that were rubbed. With endothelium present, the increase in cGMP and relaxation were related to the concentration of acetylcholine (Table 4). In contrast, rubbing the intimal surface did not alter the elevated levels of cGMP induced with nitroprusside (Table 1). Norepinephrine (0.1 μM) had no effect upon levels of cGMP (data not shown).

**TABLE 3**

<table>
<thead>
<tr>
<th>Addition</th>
<th>cGMP (pmol/mg protein)</th>
<th>Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.7 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Quinacrine (0.1 mM)</td>
<td>0.8 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Acetylcholine (10 μM) + Quinacrine (0.1 mM)</td>
<td>25.4 ± 6.6*</td>
<td>86.7 ± 8.4</td>
</tr>
<tr>
<td>Acetylcholine (10 μM)</td>
<td>6.0 ± 1.6†</td>
<td>55.5 ± 6.2†</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.8 ± 0.3</td>
<td>0</td>
</tr>
<tr>
<td>Quinacrine (0.1 mM)</td>
<td>0.8 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Nitroprusside (1 μM)</td>
<td>94.4 ± 23.7*</td>
<td>100</td>
</tr>
<tr>
<td>Nitroprusside (1 μM) + Quinacrine (0.1 mM)</td>
<td>98.8 ± 25.1*</td>
<td>100</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± se; n = 7 and 4 in experiments 1 and 2, respectively; n = 3 in all relaxation studies. Tissues were exposed to 0.1 μM norepinephrine for 5 minutes and then either 10 μM acetylcholine for 1 minute or 10 μM acetylcholine for 30 seconds, followed by 0.1 mM quinacrine for 30 seconds. Other tissues were exposed to norepinephrine followed by either 1 μM nitroprusside for 1.5 minutes or 1 μM nitroprusside for 1 minute followed by 0.1 mM quinacrine for 30 seconds. Additional tissues were exposed to norepinephrine and then 0.1 mM quinacrine for 30 seconds. Tissues were then frozen and assayed for cGMP (see Methods).

* Significantly greater than control.
† Significantly less than acetylcholine-treated tissues.

**FIGURE 1. Effect of acetylcholine on levels of cGMP, cAMP, and relaxation in rat thoracic aorta.** Aortas were rubbed (—); see Methods or allowed to remain intact (—). Tissues then were exposed to 0.1 μM norepinephrine for 5 minutes, followed by 10 μM acetylcholine for various times, frozen and assayed for cyclic nucleotides [○]; n varied from 6 to 12. Relaxation to 10 μM acetylcholine (□) was recorded in another set of tissues contracted with 0.1 μM norepinephrine (n = 8 in each case). Acetylcholine (10 μM) did not relax tissues that had been rubbed previously. cGMP and cAMP levels of rubbed tissues were significantly less than those of unrubbed tissues. * Significantly greater than control at time 0.

**FIGURE 2. Effect of histamine on levels of cGMP and relaxation in rat thoracic aorta.** Aortas were rubbed (—); see Methods or allowed to remain intact (—). Tissues then were exposed to 0.1 μM norepinephrine for 5 minutes, followed by 1 mM histamine for various times, frozen and assayed for cGMP [□]; n varied from 3 to 6. Relaxation to 1 mM histamine (○) was recorded in another set of tissues contracted with 0.1 μM norepinephrine (n = 5 in each case). Histamine (1 mM) did not relax tissues that were previously rubbed. cGMP levels of rubbed tissues were significantly less than those of unrubbed tissues. * Significantly greater than control at time 0.

**Effect of Quinacrine on cGMP Levels**

Whereas quinacrine (0.1 mM) partially reversed the elevated levels of cGMP induced by acetylcholine, it had no effect on the increased levels of cGMP induced...
by nitroprusside (Table 3). Quinacrine itself did not alter basal levels of cGMP.

Effect of 5,8,11,14-Eicosatetraynoic Acid on cGMP Levels

Pretreatment with ETYA (0.1 mM) completely prevented the increased levels of cGMP induced by acetylcholine and only partially lowered those induced by nitroprusside (Table 2). ETYA also decreased basal levels of cGMP. Lower concentrations of ETYA did not alter the relaxant effects of acetylcholine, and cGMP levels were not examined.

Effect of Rubbing on cGMP Levels after Exposure to Acetylcholine

Intact strips were incubated with 10 μM acetylcholine for 30 seconds followed by rubbing for 5 seconds in order to remove the endothelium of some tissues. Under these conditions, the cGMP levels in the residual (rubbed) strips remained elevated (Table 5).

Results are expressed as mean ± SE; n = 4 in each case. Aortas were exposed to 0.1 μM norepinephrine for 5 minutes followed by 10 μM acetylcholine for 35 seconds and then were frozen or exposed to 10 μM acetylcholine for 30 seconds, and then rubbed (see Methods) for 5 seconds while in Krebs-Ringer bicarbonate solution containing 10 μM acetylcholine and 0.1 μM norepinephrine. Tissues then were assayed for cGMP (see Methods).

* Significantly greater than control.
† Significantly less than non-rubbed tissues exposed to 10 μM acetylcholine using paired t-test.

Discussion

The present results support the hypothesis that vascular smooth muscle relaxation induced by various hormones and agents, which is dependent upon the presence of the endothelium (Furchgott and Zawadski, 1980; Furchgott, 1981), is mediated through the formation of cGMP. It has been proposed that the substance, or precursor of the substance, which is responsible for the relaxation of smooth muscle, is released from the endothelium as a result of activation of phospholipase. The present results demonstrate that quinacrine, an inhibitor of phospholipase A₂, partially reversed the relaxation induced by acetylcholine and histamine, as well as the increased levels of cGMP induced by acetylcholine. Presumably, the endothelial-derived substance can be released by A23187 independently of quinacrine, since quinacrine did not effect relaxation induced by A23187. Furchgott (1981) also observed that quinacrine had no effect on relaxation induced by A23187 in rabbit aorta. Vallee et al. (1979) suggested that quinacrine is an indirect inhibitor of phospholipase A₂ and acts by preventing Ca++ activation of phospholipase A₂. These authors observed that quinacrine had no effect on phospholipase A₂ activated by A23187 in platelets. The relaxation and increased levels of cGMP caused by nitroprusside were independent of quinacrine, since nitroprusside activates guanylate cyclase within the smooth muscle cell, probably through the generation of nitric oxide-free radical and direct activation of guanylate cyclase (Arnold et al., 1977). Quinacrine also had no effect on nitroprusside-induced increased levels of cGMP in rat ductus deferens (Spies et al., 1980).

The substance that induces relaxation is thought to have been oxidized by lipoxygenase (Furchgott and Zawadski, 1980). The present results demonstrate that an inhibitor of both lipoxygenase and cyclooxygenase, ETYA, inhibited relaxation induced by acetylcholine, histamine, and A23187, as well as the increased levels of cGMP induced by acetylcholine. ETYA partially decreased the nitroprusside-induced increased levels
Rubbing the intimal surface decreased basal levels of cGMP and cAMP. Whether this decrease is the result of the loss of the endothelial cells or the effect of some substance on the smooth muscle is not known. It is interesting to note that removal of the endothelium over a 5-second period and then freezing the tissue resulted in cGMP levels greater than basal levels (Table 5). However, after some equilibration, basal levels were decreased (Figs. 1–3 and Table 1). One might speculate that rubbing the intimal surface caused the release of some substance (arachidonic acid or some metabolite) that activates guanylate cyclase in the smooth muscle component. It is also interesting to note that an equilibration of approximately 30–60 minutes is required following rubbing before sensitivity to contractile agents returns to control levels (Furchgott and Zawadski, 1980). This equilibration may be required in order to return cGMP levels and/or subsequent steps induced by cGMP to basal levels.

Relaxation induced by acetylcholine, histamine, and A23187 was maintained over a time period in which the elevated levels of cGMP declined. A similar relationship was observed between sodium nitroprusside-induced relaxation and levels of cGMP (Rapoport and Murad, unpublished observation). The reason for this is not clear; however, several explanations are possible: (1) extrusion of cGMP into the surrounding media may have occurred; (2) phosphodiesterase activity may have increased; (3) a step(s) following the formation of cGMP which is involved in the relaxation mechanism may have a slower turnover rate than cGMP. In this regard, sodium nitroprusside-induced relaxation and protein phosphorylation were maintained over a period in which elevated cGMP levels declined (Rapoport et al., 1982). Furthermore, the sodium nitroprusside-induced protein phosphorylation profile was mimicked by 8-bromo-cGMP.

Contractile agents have also been shown to increase levels of cGMP in a variety of smooth muscle preparations (Dunham et al., 1974; Clyman et al., 1975; Diamond and Blisard, 1976; Katsuki and Murad, 1977; Kukovetz et al., 1982). Several explanations of these results are possible: (1) a cell type other than smooth muscle may be responsible for the increase in cGMP levels; (2) functionally distinct pools of cGMP may exist; and (3) cGMP may, in some instances, increase secondarily to contraction and act as a feedback inhibitor of contraction. However, in contrast to what has been reported with other blood vessels and/or species (Furchgott and Zawadski, 1980; Lee, 1980; Chand and Altura, 1981; Singer et al., 1982), acetylcholine did not elicit a contraction in rubbed or unrubbed rat thoracic aorta.

The increased levels of cGMP observed in these studies presumably occurred within the smooth muscle cells, since increased cGMP levels were still observed when the endothelium was removed during exposure to acetylcholine. These studies support the hypothesis that the relaxant effects of agents which require the presence of endothelium may also be mediated through the accumulation of cGMP, as appear to be the effects of nitrovasodilators. The nitrovasodilators may, in fact, act through bypassing part of the endothelium-dependent vasodilator system and directly activating guanylate cyclase in smooth muscle. Altered cGMP formation may also play an important role in vascular contraction and spasm in various pathophysiological states with endothelial injury.

The skillful technical assistance of Gilberto A. Martinez is gratefully appreciated. We would also like to thank Dr. Jon Kosek for the scanning electron microscopic studies and Kathleen McFadden for help in preparation of the manuscript.

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INDEX TERMS: Vascular smooth muscle-Endothelium-Relaxation-Cyclic guanosine monophosphate-Vasodilators
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R M Rapoport and F Murad

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