Mechanisms of Adenosine Triphosphate-, Thrombin-, and Trypsin-induced Relaxation of Rat Thoracic Aorta

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SUMMARY. The mechanisms by which adenosine triphosphate, thrombin, and trypsin cause relaxation of vascular smooth muscle were investigated. Relaxation of the rat thoracic aorta with adenosine triphosphate, thrombin, and/or trypsin was associated with increased levels of cyclic guanosine monophosphate in both time- and concentration-dependent manners. Thrombin and trypsin did not alter cyclic adenosine monophosphate levels, whereas adenosine triphosphate increased cyclic adenosine monophosphate levels after significant relaxation occurred. Removal of the endothelium abolished adenosine triphosphate-, thrombin-, and trypsin-induced relaxation and the associated increased levels of cyclic nucleotides. Relaxation due to these agents was also inhibited by exposure to nordihydroguaiaretic acid, a lipoxygenase inhibitor, and eicosatetraynoic acid, a lipoxygenase and cyclooxygenase inhibitor. Indomethacin, a cyclooxygenase inhibitor, potentiated relaxation to these agents, whereas the increased levels of cyclic nucleotides due to adenosine triphosphate were unaltered. Removal of extracellular calcium, which also presumably inhibits phospholipase A2, prevented the elevated levels of cyclic nucleotides and the inhibitory effects of adenosine triphosphate and trypsin on contraction. In contrast, sodium nitroprusside-induced relaxation and/or increased levels of cyclic guanosine monophosphate were unaltered by nordihydroguaiaretic acid, eicosatetraynoic acid, bromophenacyl bromide, and removal of extracellular calcium. After incubation of intact tissue with [32P]-orthophosphate, the patterns of protein phosphorylation caused by adenosine triphosphate, thrombin, and trypsin were indistinguishable from those of acetylcholine, sodium nitroprusside and 8-bromo cyclic guanosine monophosphate. All these agents dephosphorylated myosin light chain. Thus, the present study supports the hypothesis that relaxation induced by adenosine triphosphate, thrombin, and trypsin is mediated through the formation of an endothelial factor which elevates cyclic guanosine monophosphate levels and causes cyclic guanosine monophosphate-dependent protein phosphorylation and dephosphorylation of myosin light chain. (Circ Res 55: 468-479, 1984)

ENDOTHELIUM-dependent vasodilation induced by acetylcholine, histamine and Ca++ ionophore A23187 has recently been shown to be associated with the formation of cyclic guanosine monophosphate (cGMP) in both a time- and concentration-dependent manner in rat thoracic aorta (Rapoport and Murad, 1983a). Furthermore, an inhibitor of phospholipase A2, quinacrine, and an inhibitor of lipoxygenase and cyclooxygenase, eicosatetraynoic acid (ETYA), inhibited acetylcholine-induced relaxation and the formation of cGMP (Rapoport and Murad, 1983a). These results confirmed those of others (Furchgott and Zawadzki, 1980a; Furchgott et al., 1981; Furchgott, 1983) which suggested that relaxation caused by these agents was through activation of phospholipase A2 and subsequent release of an unsaturated fatty acid. The fatty acid presumably is oxidized by lipoxygenase, and the unknown product activates guanylate cyclase within the smooth muscle and increases levels of cGMP. It has been hypothesized that the elevated cGMP levels may then induce relaxation (Katsuki et al., 1977; Katsuki and Murad, 1977; Schultz et al., 1977).

We have also recently reported that the pattern of protein phosphorylation induced by acetylcholine in intact rat aorta is identical to that caused by sodium nitroprusside and 8-bromo cGMP (Rapoport et al., 1982, 1983a). Furthermore, the changes in phosphorylation caused by acetylcholine were dependent on an intact endothelium, whereas those induced by sodium nitroprusside and 8-bromo cGMP were endothelium independent.

Vasodilation with thrombin or adenosine triphosphate (ATP) has also been shown to be dependent on the presence of the endothelium in canine femoral, pulmonary, saphenous, and splenic arteries and veins (De Mey and Vanhoutte, 1981, 1982; De Mey et al., 1982), coronary arteries (Ku, 1982), and rabbit aorta (Furchgott, 1981). However, in contrast to the relaxations induced by acetylcholine, relaxation of the canine femoral artery with thrombin or ATP was not inhibited by ETYA or quinacrine (De...
Mey et al., 1982). Thus, the mechanisms by which thrombin and ATP induce endothelium-dependent vasodilation in the canine femoral artery are thought to be different from that of norepinephrine (De Mey et al., 1982). However, ATP-induced relaxation was inhibited by ETYA or quinacrine in rabbit aorta (Furchgott, 1981).

The purpose of the present study was to investigate the mechanism by which thrombin and ATP cause vasodilation in rat thoracic aorta. The effects of ATP, thrombin, and another protease, trypsin, on relaxation and on the formation of cGMP and cyclic adenosine monophosphate (cAMP) were investigated in the rat thoracic aorta with and without endothelium. The effects of inhibitors of phospholipase A2, cyclooxygenase, and lipoxygenase and removal of extracellular Ca++ on endothelium-dependent relaxation and cyclic nucleotide levels were also investigated and compared to responses elicited by nitroprusside. The pattern of protein phosphorylation with these agents was compared to that resulting from another endothelium-dependent vasodilator, acetylcholine, as well as to sodium nitroprusside and 8-bromo cGMP. A preliminary account of some of these observations has been previously published (Rapoport et al., 1983b).

### Methods

#### Relaxation Studies

Rats (Sprague-Dawley, male, 240–300 g) were decapitated, their thoracic aortas removed and cut into spiral strips (approximately 2 mm x 1 cm). Care was taken not to allow the lumen to come into contact with any solid surface. The strips then were mounted in organ baths containing Krebs-Ringer bicarbonate solution which was gassed with 95% O2-5% CO2 and which 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 0.2 g-force was maintained throughout the experiment. Tissues were left to equilibrate for 2 hours before the addition of any drugs.

Contraction was elicited with 0.1 μM norepinephrine, which induced approximately 80% of the maximum contractile response to norepinephrine, followed by the addition of ATP, thrombin, trypsin, or sodium nitroprusside. The intimal surface of some of the strips was gently rubbed with a scalpel, which removed the endothelial layer and left the internal elastic lamina intact, as confirmed with scanning electron microscopy (Rapoport and Murad, 1983a). Following reequilibration (30–60 minutes), subsequent contractile responses to 0.1 μM norepinephrine were unaffected by the rubbing procedure. Other strips were pretreated with ETYA (0.1 mM, 30 minutes), nordihydroguaiaretic acid (10 μM, 60 minutes), indomethacin (0.1 mM, 60 minutes), or bromophenacyl bromide (3 μM, 60 minutes), followed by the addition of 0.1 μM norepinephrine and the smooth muscle relaxants for the appropriate times. Tissues were washed with fresh Krebs-Ringer bicarbonate solution after exposure to bromophenacyl bromide.

The concentration and time of exposure to ETYA previously has been used in other studies investigating endothelium-dependent relaxation (Furchgott and Zawadzki, 1980; Furchgott et al., 1981; Furchgott, 1983). The concentrations of nordihydroguaiaretic acid and bromophenacyl bromide are lower than those used in prior studies, although the exposure times are somewhat longer (Furchgott et al., 1982; Furchgott, 1983). It was found that nordihydroguaiaretic acid and bromophenacyl bromide inhibited the endothelium-dependent relaxations to a greater degree at lower concentrations, when longer preincubation periods were used (data not shown). Indomethacin was used at a slightly greater concentration than that employed by others (Furchgott and Zawadzki, 1980; Furchgott et al., 1981; DeMey et al., 1982; Furchgott, 1983), although others have also exposed tissues to indomethacin for 60 minutes prior to testing endothelium-dependent relaxation (DeMey et al., 1982). The concentration and time of exposure to indomethacin were maximized to eliminate any possibility of cyclooxygenase involvement in endothelium-dependent relaxation or elevated cGMP levels.

The effects of Ca++-free Krebs-Ringer bicarbonate solution on the ATP-, trypsin-, and sodium nitroprusside-induced elevated levels of cGMP and/or cAMP and on the ability of these agents to inhibit the contractile response to norepinephrine were investigated. To test the effects of these agents on the contractile response, tissues were washed three times with Ca++-free Krebs-Ringer bicarbonate solution containing 2 mM EGTA over a 12-minute period. Additional tissues were exposed to ATP, trypsin, or sodium nitroprusside for the last 2 minutes.

Tissues were then exposed to 0.3 μM norepinephrine. The observed contractile response was rapid and phasic in nature (Fig. 1k). A higher concentration of norepinephrine was used in these studies, since a greater contractile response was elicited which could be accurately quantified (data not shown). Cyclic nucleotide levels were measured after 10 minutes of exposure to Ca++-free Krebs-Ringer bicarbonate solution, as above, followed by ATP or trypsin for 30 seconds or sodium nitroprusside for 1.5 minutes [times of maximal levels of cGMP (present study and Rapoport et al., 1983b)]. Tissues then were frozen and assayed for cyclic nucleotides (see below).

As additional controls, the effects of prior exposure to ATP, trypsin, or sodium nitroprusside on the contractile response to 0.3 μM norepinephrine and alterations in cyclic nucleotide levels were tested in normal Krebs-Ringer bicarbonate solution. The response to norepinephrine consisted of an initial rapid contraction followed by a slow tonic contraction, which were more apparent at the greater norepinephrine concentration (Fig. 1j). The initial rapid contraction is thought to be due, at least in part, to the same source of Ca++ as the rapid, transient contraction elicited in Ca++-free Krebs-Ringer bicarbonate solution (for a review, see Bolton, 1979, and references therein). Thus, we could test the effects of ATP, trypsin, and sodium nitroprusside on the rapid, initial contraction observed in normal Krebs-Ringer bicarbonate solution, as well as in Ca++-free solution, and also their effects on cyclic nucleotide levels under these two conditions. Tissues were exposed to ATP, trypsin, and sodium nitroprusside in normal Krebs-Ringer bicarbonate solution at the same concentrations and times as in experiments performed with Ca++-free Krebs-Ringer bicarbonate solution.

#### cGMP and cAMP Measurements

Rubbed or unrubbed spiral strips were prepared as described above and were allowed to equilibrate in flasks.
PAIRING MEANS

All means that were significantly different by unpaired Student's t-test were also significantly different by paired t-test. Those means that are indicated as significantly different by paired t-test were not significantly different by unpaired t-test. Analysis of variance was also performed as indicated.

Protein Phosphorylation Studies

These studies were performed essentially as previously described (Rapport et al., 1982, 1983a; Draznin et al., 1983). Briefly, spiral strips from 13 thoracic aortas were cut into five segments approximately 1.5 cm long. Segments from each of the 13 aortas (approximately 13 mg total protein) were incubated in one of five flasks containing 1.5 ml of phosphate-free Krebs-Ringer bicarbonate solution under an atmosphere of 95% O2-5% CO2. Two to 2.5 mCi of carrier free 32P-orthophosphoric acid were added to each flask and tissues incubated for 2 hours at 37°C in a shaking water bath. Tissues were exposed to 0.3 μM norepinephrine for 3 minutes, with or without agents for the last 30 seconds, and the tissues rapidly removed from the incubation buffer and frozen in liquid nitrogen. The strips were then homogenized in ice-cold buffer containing 100 mM NaF, 80 mM sucrose, 10 mM EDTA, 10 mM N-ethylmaleimide, 1% trypsin, 1% benzamidine, and 50 μM/ml each of soybean trypsin inhibitor, leupeptin, aprotinin, and ovomucoid trypsin inhibitor. Prior to electrophoresis, aliquots of the samples were assayed for protein concentration (Lowry et al., 1951) and adjusted with homogenization buffer so that protein concentrations were equivalent in all samples.

Samples were heated (98°C) in 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 10% glycerol for 15 minutes, cooled, and urea, Nonidet P-40, and ampholines were added to yield final concentrations of 9.5 M, 9% and 2% (vol/vol), respectively, after the method of Anderson and Anderson (1978). The ratio of ampholines, pH 3-10: pH 5-10 was 1:2. Two hundred to 800 μg of protein were loaded onto each 0.3 X 19.5 cm tube gel containing 3% acrylamide, 2% Nonidet P-40, and ampholines (type I-S), thrombin (bovine, 200 NIH U/mg), and trypsin (type I) plus antithrombin (Anti-thr), followed by (f) 3 U/ml thrombin and again to (g) 3 U/ml thrombin. In another tissue segment, contractions were elicited to (h) 0.3 μM norepinephrine, which consisted of a rapid, initial contraction followed by a slow, tonic contraction (the arrow shows the approximate point where the initial phase ends and the tonic phase begins) and, in the same segment, (i) the response to 0.3 μM norepinephrine after exposure to Ca**+-free Krebs-Ringer bicarbonate solution for 12 minutes (see Methods).

FIGURE 1. Rat thoracic aortas were exposed to (a) 0.1 μM norepinephrine followed by 10 μM ATP or (b) 0.1 μM ATP. This same tissue was then exposed to (c) 25 μg/ml trypsin and then again to (d) 25 μg/ml trypsin followed by 0.1 μM ATP. A paired tissue (e) was rubbed in order to remove the endothelium and exposed to 25 μg/ml trypsin, while another rubbed tissue (f) was exposed to 0.1 μM and 0.1 mM ATP. An additional aorta segment was exposed to (g) 3 U/ml thrombin (Thr) plus antithrombin (Anti-thr), followed by (h) 3 U/ml thrombin and again to (i) 0.3 U/ml thrombin. In another tissue segment, contractions were elicited to (j) 0.3 μM norepinephrine, which consisted of a rapid, initial contraction followed by a slow, tonic contraction (the arrow shows the approximate point where the initial phase ends and the tonic phase begins) and, in the same segment, (k) the response to 0.3 μM norepinephrine after exposure to Ca**+-free Krebs-Ringer bicarbonate solution for 12 minutes (see Methods).

containing Krebs-Ringer bicarbonate solution gassed with 95% O2-5% CO2. At 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 the homogenates centrifuged. Supernatant fractions were extracted with ether, acetylated (Harper and Brooker, 1975), and radioimmunoassayed for cyclic nucleotides (Steiner et al., 1972). Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Statistical significance was accepted at the 0.05 level of probability using unpaired and paired Student's t-test. All means that were significantly different by unpaired t-test were also significantly different by paired t-test. Those means that are indicated as significantly different by paired t-test were not significantly different by unpaired t-test.

Materials

Acetylcholine-HCl, adenosine 5'-triphosphate (sodium salt), antithrombin III (bovine, 400–600 U/mg), aprotinin, benzamidine, 4-bromophenacyl bromide, hirudin (grade IV, 1000–1500 U/mg), indomethacin, leupeptin, Nonidet P-40, nordihydroguaiaretic acid, l-norepinephrine-HCl, ovomucoid trypsin inhibitor, 1,10-phenanthroline, phenylmethylsulfonfluoride, plasmid (100 U/mg), sodium bisulfite, sodium nitroprusside, soybean trypsin inhibitor (type I-S). Thrombin (bovine, 200 NIH U/mg), and trypsin (type I, 11,700 BAAE U/mg) were obtained from Sigma. Carrier free 32P-orthophosphoric acid was obtained from ICN, urea (ultrapure) from Schwarz-Mann, ampholines
from Bio-Rad, and 5,8,11,14-eicosatetraynoic acid (ETYA) was a gift from Hoffman-La Roche. Other materials were obtained as previously described (Katsuki et al., 1977; Katsuki and Murad, 1977).

Results

Effect of Agents and Removal of the Endothelium on ATP-, Thrombin-, and Trypsin-induced Relaxation

The effect of various inhibitors of arachidonic acid metabolism and removal of the endothelium on relaxations induced by ATP, thrombin, and trypsin are summarized in Table 1. The concentrations of thrombin (3 U/ml) and trypsin (25 μg/ml) were maximally effective at causing relaxation, whereas 10 μM ATP gave approximately 80% of the maximal relaxation to ATP (Table 1; Fig. 1a, and see below). This concentration of ATP (10 μM) was used, since relaxations with higher concentrations of ATP induced a rapid, endothelium-dependent relaxation, followed by a slower, endothelium-independent relaxation, which, in some experiments, completely relaxed the tissue (Fig. 1, b, d, and f). The endothelium-independent relaxation induced by 10 μM ATP was of a much smaller magnitude or absent (Fig. 1, a and f).

Some relaxations induced by 10 μM ATP were not as well sustained as that shown in Figure 1a. The endothelium-independent relaxations due to ATP were not inhibited by indomethacin (data not shown).

Relaxations induced by ATP, thrombin, and trypsin were abolished in tissue without endothelium (Table 1; Fig. 1, e and f). ETYA, an inhibitor of lipoxygenase and cyclooxygenase (Flower, 1974), and nordihydroguaiaretic acid, a lipoxygenase inhibitor (Hamberg, 1976; Goetzl et al., 1980), inhibited relaxations due to these agents. In contrast, sodium nitroprusside-induced relaxations in control and tissues pretreated with ETYA were not significantly different (8.63 ± 0.08 and 8.58 ± 0.05; 8.38 ± 0.09 and 8.28 ± 0.04, respectively; −logEC50 and −logEC30 ± SE, n = 6 in each case). Nordihydroguaiaretic acid had no significant effect on relaxations induced by sodium nitroprusside (8.56 ± 0.08 and 8.62 ± 0.17; 8.33 ± 0.08 and 8.41 ± 0.18, −logEC50 and −logEC30 ± SE in control and nordihydroguaiaretic acid-treated tissues, respectively; n = 6 in each case). Indomethacin, a cyclooxygenase inhibitor (Flower, 1974), potentiated the endothelium-dependent relaxations (Table 1), as well as the relaxations to sodium nitroprusside and isoproterenol (data not shown). Bromophenacyl bromide, an inhibitor of phospholipase (Robert et al., 1977; Vallee et al., 1979; Thakker et al., 1983), inhibited the endothelium-dependent relaxations. The contraction responses to 0.1 μM norepinephrine after pretreatment with 0.1 mM ETYA, 10 μM nordihydroguaiaretic acid, and 0.1 mM indomethacin were significantly reduced to 71.2 ± 3.6, 46.4 ± 3.0, and 53.7 ± 4.2% (mean ± SE) of control, respectively, while the responses to norepinephrine following 3 μM bromophenacyl bromide pretreatment were 94.8 ± 13.9% (mean ± SE) of control. Relaxations were expressed as percent of the contraction observed following addition of these agents. Bromophenacyl bromide usually elicited a transient contraction.

The relaxations induced by thrombin and trypsin were transient in nature and reached a maximum response by 30 seconds (Fig. 1, c and h). After the relaxation, the tissue sometimes contracted to a height greater than that of the initial plateau contraction induced by 0.1 μM norepinephrine. Plasmin (150 μg/ml) had no effect on the contraction to norepinephrine (data not shown).

Thrombin-induced relaxations were abolished by preincubation of thrombin with an approximate 1.5-fold molar excess of antithrombin III for 1 hour (Fig. 1g) or 1.5 U of hirudin: 1 U of thrombin for 15 minutes in Krebs-Ringer bicarbonate solution. Prior exposure of tissues to thrombin-antithrombin complex, or to antithrombin followed by a wash, had no effect on subsequent relaxation to thrombin (data not shown). Trypsin-induced relaxation was abolished by the addition of an equivalent amount (weight:weight) of soybean trypsin inhibitor, whereas a 100-fold excess amount of soybean trypsin inhibitor had no effect on thrombin-induced relaxation.

![Table 1](image)

**Table 1**

Effect of Agents and Removal of the Endothelium on ATP-, Thrombin-, and Trypsin-induced Relaxation in Rat Thoracic Aorta

<table>
<thead>
<tr>
<th>Relaxant</th>
<th>Control</th>
<th>Without endothelium</th>
<th>Eicosatetraynoic acid</th>
<th>Nordihydroguaiaretic acid</th>
<th>Indomethacin</th>
<th>Bromophenacyl bromide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (10 μM)</td>
<td>54.9 ± 3.9 (6)</td>
<td>0* (9)</td>
<td>0* (3)</td>
<td>6.5 ± 3.3* (3)</td>
<td>82.7 ± 9.6† (3)</td>
<td>7.8 ± 3.8* (6)</td>
</tr>
<tr>
<td>Thrombin (3 U/ml)</td>
<td>29.5 ± 3.6 (17)</td>
<td>0* (6)</td>
<td>0* (3)</td>
<td>0* (3)</td>
<td>50.7 ± 5.0† (4)</td>
<td>0* (6)</td>
</tr>
<tr>
<td>Trypsin (25 μg/ml)</td>
<td>73.5 ± 3.0 (20)</td>
<td>0* (6)</td>
<td>8.3 ± 0.8* (4)</td>
<td>3.4 ± 2.1* (4)</td>
<td>91.9 ± 6.1† (6)</td>
<td>4.3 ± 2.5* (8)</td>
</tr>
</tbody>
</table>

Some aortas were exposed to either 0.1 mM eicosatetraynoic acid for 30 minutes, 10 μM nordihydroguaiaretic acid, 0.1 mM indomethacin, or 3 μM bromophenacyl bromide for 60 minutes prior to exposure to 0.1 μM norepinephrine. Bromophenacyl bromide was washed from the tissue bath prior to addition of norepinephrine. The intimal surface of additional strips was rubbed in order to remove the endothelium (see Methods). Results are expressed as percent relaxation of the contractile response (mean ± SE, n).

*Significantly less than control.
†Significantly more than control.
relaxation. Antithrombin III, hirudin, and soybean trypsin inhibitor had no effect on the contraction induced by 0.1 μM norepinephrine.

Effect of ATP on Cyclic Nucleotide Levels and Relaxation

Exposure of aorta strips contracted with 0.1 μM norepinephrine to 1.0, 10, and 100 μM ATP induced 7.4 ± 0.9, 54.9 ± 3.9, and 66.9 ± 2.8% relaxation, respectively (mean ± SE, n = 6 in each case; measured approximately 30 seconds after addition; see Fig. 1). ATP (10 μM) exposure for 30 seconds to 0.1 μM norepinephrine contracted tissues significantly elevated cGMP levels from 0.7 ± 0.2 to 6.1 ± 2.5 pmol/mg protein and cAMP levels from 1.4 ± 0.3 to 2.4 ± 0.2 pmol/mg protein (mean ± SE, n = 5 in each case). ATP (100 μM) induced further elevations of cGMP and cAMP levels (Table 2). Thus, increased amounts of relaxation were associated with increased levels of cyclic nucleotides. Removal of the endothelium abolished the ATP-induced increased levels of cyclic nucleotides and relaxation (Tables 1 and 2; Fig. 1). Basal levels of cyclic nucleotides were also decreased in tissues that had their endothelium removed (Table 2).

Increased levels of cGMP and cAMP occurred within 5 and 10 seconds, respectively, after exposure to 0.1 mM ATP (Fig. 2). Exposure of tissues to 0.1 mM ATP for 5 seconds induced 9.5 ± 1.4% (mean ± SE, n = 8) relaxation of the contractile response to 0.1 μM norepinephrine. Peak relaxation due to 0.1 mM ATP occurred approximately 30 seconds after exposure, although the endothelium-independent component of the relaxation adds some amount of error to this determination (Fig. 1, b, d, and f). Maximum levels of cGMP and cAMP were observed 30 and 10 seconds, respectively, after exposure to 0.1 mM ATP (Fig. 2). The elevated level of cGMP then decreased over the next 30 minutes, while the cAMP level remained elevated.

Effect of Thrombin and Trypsin on Cyclic Nucleotide Levels

Relaxation induced by thrombin and trypsin was associated with increased levels of cGMP, whereas cAMP levels were unaltered (Tables 1 and 2). Removal of the endothelium abolished thrombin- and trypsin-induced increased levels of cGMP and relaxations. Basal levels of cGMP and cAMP were decreased in tissues that had their endothelium removed.

Effect of Bromophenacyl Bromide on Cyclic Nucleotide Levels and Relaxation Induced by ATP, Thrombin, and Sodium Nitroprusside

Bromophenacyl bromide (3 μM) inhibited ATP- and thrombin-induced increased levels of cGMP and relaxation (Tables 1 and 3). Endothelium-dependent relaxations induced by 0.1 mM ATP were reduced by bromophenacyl bromide from 66.9 ±
by bromophenacyl bromide (data not shown). Basal levels of cGMP were decreased by bromophenacyl bromide. The effect of bromophenacyl bromide on relaxation and cyclic nucleotide levels was investigated; rather than quinacrine, another phosphodiesterase A2 inhibitor which we previously used (Rapoport and Murad, 1983a), since bromophenacyl bromide, unlike quinacrine, did not alter the contractile response to norepinephrine (see above and Rapoport and Murad, 1983a).

**Effect of ATP, Trypsin, and Sodium Nitroprusside in the Absence of Extracellular Ca**++ **on Cyclic Nucleotide Levels and on Norepinephrine-Induced Contraction**

Removal of extracellular Ca++ decreased the magnitude of the ATP- and trypsin-induced increased levels of cGMP, whereas those due to sodium nitroprusside were unaffected (Table 4). The elevated levels of cAMP induced by ATP were also decreased. Basal levels of cGMP were reduced in tissues exposed to Ca++-free Krebs-Ringer bicarbonate solution.

Pretreatment with ATP reduced the magnitude of both the phasic and tonic contractions induced by norepinephrine in normal Krebs-Ringer bicarbonate solution (Fig. 1j; Table 4). The phasic contraction elicited in Ca++-free Krebs-Ringer bicarbonate solution (Fig. 1k) was only slightly decreased by pretreatment with ATP. In contrast, pretreatment with sodium nitroprusside almost completely inhibited the phasic contractions induced by norepinephrine in both normal and Ca++-free Krebs-Ringer bicarbonate solution. The phasic contractions induced by norepinephrine in Ca++-free Krebs-Ringer bicarbonate solution were significantly less than those elicited in normal Krebs-Ringer bicarbonate solution (Table 4). The effect of prior exposure to trypsin on the norepinephrine-induced contractile responses and cyclic nucleotide levels in normal and Ca++-free Krebs-Ringer bicarbonate solution was not tested, since trypsin caused a transient relaxation (Fig. 1c).

**Effect of Indomethacin on ATP-induced Increased Levels of Cyclic Nucleotides**

Pretreatment with indomethacin, a cyclooxygenase inhibitor (Flower, 1974), had no effect on ATP-induced increased levels of cGMP and cAMP (Table 5). Basal levels of cGMP were increased by indomethacin.

**Effect of Thrombin on cGMP Levels and Relaxation in Thrombin-Desensitized Tissues**

Desensitization to the relaxant effects of thrombin were induced by prior exposure to thrombin or trypsin and vice versa (Fig. 1, c, d, h, and i, and Table 6; some data not shown). Thrombin-induced increased levels of cGMP and relaxation were abolished in tissues pretreated with thrombin (Table 6).
TABLE 4

Effect of ATP, Trypsin, and Sodium Nitroprusside on Cyclic Nucleotide Levels and Norepinephrine-Induced Contraction in Normal and Ca**-Free Krebs-Ringer Bicarbonate Solution in Rat Thoracic Aorta

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pmol/mg protein</th>
<th>Norepinephrine contraction (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cGMP</td>
<td>cAMP Phasic Tonic</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.9 ± 0.1</td>
<td>0.38 ± 0.03 (10) 0.79 ± 0.07 (12)</td>
</tr>
<tr>
<td>Ca**-free solution</td>
<td>0.3 ± 0.1*</td>
<td>2.1 ± 0.4 0.26 ± 0.02* (12)</td>
</tr>
<tr>
<td>+ ATP (0.1 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca**-free solution</td>
<td>30.9 ± 3.2†</td>
<td>5.1 ± 0.6† 0.13 ± 0.01* (10) 0.18 ± 0.02* (12)</td>
</tr>
<tr>
<td>+ ATP (0.1 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATp (0.1 mM)</td>
<td>9.2 ± 2.2††</td>
<td>3.8 ± 0.7†† 0.22 ± 0.02†† (12)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.3 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>Ca**-free solution</td>
<td>0.3 ± 0.1*</td>
<td>2.2 ± 0.7 ND</td>
</tr>
<tr>
<td>+ EGTA (2 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin (25 µg/ml)</td>
<td>48.6 ± 3.0†</td>
<td>3.2 ± 0.5 ND</td>
</tr>
<tr>
<td>Ca**-free solution</td>
<td>6.3 ± 2.2††</td>
<td>3.3 ± 0.8 ND</td>
</tr>
<tr>
<td>+ EGTA (2 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ trypsin (25 µg/ml)</td>
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<tr>
<td>Experiment 3</td>
<td></td>
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</tr>
<tr>
<td>None</td>
<td>1.3 ± 0.2</td>
<td>0.46 ± 0.03 (6) 0.86 ± 0.08 (6)</td>
</tr>
<tr>
<td>Ca**-free solution</td>
<td>0.8 ± 0.1*</td>
<td>3.6 ± 0.3 0.29 ± 0.03 (3)</td>
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<tr>
<td>+ EGTA (2 mM)</td>
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<td></td>
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<tr>
<td>Sodium nitroprusside (0.1 µM)</td>
<td>5.3 ± 1.2†</td>
<td>3.3 ± 0.8 0.07 ± 0.01* (6) 0.10 ± 0.02* (6)</td>
</tr>
<tr>
<td>Ca**-free solution</td>
<td>5.2 ± 0.8††</td>
<td>3.0 ± 0.2 0.03 ± 0.01†† (3)</td>
</tr>
<tr>
<td>+ EGTA (2 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ sodium nitroprusside (0.1 µM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In experiments in which cyclic nucleotide levels were determined, aortas were exposed to 0.1 mM ATP or 25 µg/ml trypsin for 30 seconds or 0.1 µM sodium nitroprusside for 1.5 minutes (time of maximal level of cGMP) in normal Krebs-Ringer bicarbonate solution or after 10 minutes of exposure to Ca**-free solution containing 2 mM EGTA (see Methods). Additional tissues remained unexposed to ATP, trypsin, or sodium nitroprusside. Tissues were then frozen and assayed for cyclic nucleotides (see Methods). In experiments in which contractile responses were determined, aortas were exposed to 0.1 mM ATP or 0.1 µM sodium nitroprusside for 2 minutes (plateau relaxation) in normal Krebs-Ringer bicarbonate solution or after 10 minutes of exposure to Ca**-free solution containing EGTA (see Methods). Additional tissues remained unexposed to ATP or sodium nitroprusside. Tissues were then contracted with 0.3 µM norepinephrine and their phasic and tonic responses recorded (see Methods and Figure 1). Results are expressed as mean ± SE. n = 7, 4, and 3 in cyclic nucleotide measurements in experiments 1, 2, and 3, respectively. In contraction experiments, n is shown for each experimental condition. Means were significantly different in cGMP and contraction measurements in experiments 1, 2, and 3 and cAMP measurements in experiment 1, using analysis of variance. ND = not determined.

* Significantly less than control.
† Significantly more than control.
‡ Significantly less than ATP- and trypsin-treated tissues.
§ Significantly less than ATP-treated tissues, paired t-test.
|| Significantly less than tissues exposed to Ca**-free solution, paired t-test.
|| Significantly less than tissues exposed to Ca**-free solution.

In contrast, relaxations to ATP and acetylcholine were unaltered in tissues desensitized to thrombin and trypsin (Fig. 1, b and d; some data not shown). We then tested whether the elevated cGMP levels caused by ATP were altered in tissues desensitized to trypsin. The elevated levels of cGMP induced by ATP were not different in tissues desensitized to trypsin as compared to controls (Table 7).

Effect of ATP, Thrombin, and Trypsin on Protein Phosphorylation

Treatment of aorta strips with ATP (0.1 mM), thrombin (3 U/ml), or trypsin (25 µg/ml) increased 32P incorporation into the following proteins (molecular weight in kdaltons, p): 49.0, 7.8; 49.0, 7.4; 49.0, 7.1; 28.0, 6.8; 25.0, 6.7; 24.0, 6.7; 24.0, 6.5; 24.0, 6.2; 21.5, 6.6; 21.3, 6.4; 21.3, 6.4, and de-
Increased 32P incorporation into other proteins (kda-
tons; pl: 20.0, 5.3 and 20.0, 5.1. This pattern of
protein phosphorylation was identical to that ob-
served for sodium nitroprusside and 8-bromo cGMP
in tissues with or without endothelium and to ace-
tylcholine and Ca++ ionophore A23187 in tissues
with intact endothelium (Rapoport et al., 1982,
1983a). The two proteins of 20 kda, which were
dephosphorylated following treatment with ATP,
thrombin, trypsin, acetylcholine, sodium nitroprus-
side, or 8-bromo cGMP, have been identified as
myosin light chain (Rapoport et al., 1982). Norepi-
nehpinephrine (0.3 μM) increased the level of myosin light

**TABLE 5**

<table>
<thead>
<tr>
<th>Addition</th>
<th>cGMP (pmol/mg protein)</th>
<th>cAMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.7 ± 0.1</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>Indomethacin (0.1 mM)</td>
<td>1.1 ± 0.1*</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>ATP (0.1 mM)</td>
<td>16.8 ± 2.0*</td>
<td>5.0 ± 0.6*</td>
</tr>
<tr>
<td>Indomethacin (0.1 mM) + ATP (0.1 mM)</td>
<td>19.5 ± 3.0*</td>
<td>4.9 ± 0.6*</td>
</tr>
</tbody>
</table>

**TABLE 6**

<table>
<thead>
<tr>
<th>Addition</th>
<th>cGMP (pmol/mg protein)</th>
<th>Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine (0.1 μM)</td>
<td>0.4 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Norepinephrine (0.1 μM) + thrombin (3 U/ml)</td>
<td>1.2 ± 0.3*</td>
<td>31.3 ± 1.2*</td>
</tr>
<tr>
<td>Norepinephrine (0.1 μM), then norepinephrine (0.1 μM)</td>
<td>0.5 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Norepinephrine (0.1 μM) + thrombin (3 U/ml), then norepinephrine (0.1 μM)</td>
<td>0.5 ± 0.2†</td>
<td>0†</td>
</tr>
</tbody>
</table>

**TABLE 7**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cGMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Trypsin (25 μg/ml)</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>ATP (0.1 mM)</td>
<td>19.6 ± 2.2*</td>
</tr>
<tr>
<td>Trypsin (25 μg/ml) + ATP (0.1 mM)</td>
<td>18.9 ± 5.1*</td>
</tr>
</tbody>
</table>

**Discussion**

We have previously shown that endothelium-
dependent relaxations induced by acetylcholine, his-
tamine, and Ca++ ionophore A23187 in rat thoracic
aorta may be mediated through the formation of
cGMP (Rapoport and Murad, 1983a). The suggested
mechanism by which the endothelium-dependent
relaxation occurred was consistent with that of
Furchgott et al. (1981). That is, the endothelium-
dependent vasodilators activate phospholipase A2
within the endothelial cells. The activation causes
release of an unsaturated fatty acid, which is then
oxidized by lipoygenase. Unsaturated fatty acids,
hydroxyl free radicals, and lipid peroxides have been
shown to activate guanylate cyclase, directly (Wal-
ach and Pastan, 1976; Glass et al., 1977; Hidaka and
Asano, 1977; Mittal and Murad, 1977). Thus, the
fatty acid and/or the possible free radicals formed
by the oxidized fatty acid may activate guanylate
cyclase and elevate levels of cGMP. The increased
levels of cGMP may then activate cGMP-dependent
protein kinase (Fiscus et al., in press), resulting in
protein phosphorylation and dephosphorylation of
myosin light chain (Rapoport et al., 1982, 1983a)
and smooth muscle relaxation. The present study
extends this hypothesis to three other endothelium-
dependent relaxants, ATP, thrombin, and trypsin.

ATP-induced relaxation was associated with in-
creased levels of cGMP in both a time- and concen-
tration-dependent manner. Removal of the endothe-
lium abolished the increased cGMP levels and the
relaxations to ATP, thrombin, and trypsin. These
agents presumably activate phospholipase A2, since
bromophenacil bromide, a phospholipase A2 inhib-
itor (Roberts et al., 1977; Vallee et al., 1979; Thakkar et al., 1983), inhibited increased cGMP levels and relaxation induced by ATP and thrombin, whereas increased levels of cGMP and relaxation caused by sodium nitroprusside, an endothelium-independent relaxant (Rapoprt and Murad, 1983a, 1983b), were unaltered. Bromophenacyl bromide has also been shown to inhibit endothelium-independent relaxation to acetylcholine and Ca++ ionophore A23187 in rabbit aorta and canine arteries (Furchgott et al., 1982).

Removal of extracellular Ca++, which decreases phospholipase A2 activity (Eisenberg et al., 1968; Flower and Blackwell, 1976; Thakker et al., 1983), as well as having other effects, reduced ATP-induced increased levels of cGMP and decreased the inhibition of the norepinephrine-induced contraction, and had no effect on the sodium nitroprusside-induced increased levels of cGMP and inhibition of contraction. Others have also shown that sodium nitroprusside-induced increased levels of cGMP are independent of extracellular Ca++ (Katsuki and Murad, 1977; Schultz et al., 1977; Spies et al., 1980), and that sodium nitroprusside-induced inhibition of the norepinephrine contraction was unaffected by removal of extracellular Ca++ (Verhaeghe and Shepherd, 1976; Kreye, 1980; Heaslip and Rahman, 1983; Lincoln, 1983). Methacholine- and Ca++ ionophore A23187-induced endothelium-dependent relaxations of rabbit thoracic aorta were also inhibited by removal of extracellular Ca++ or by addition of Ca++ antagonists (Singer and Peach, 1982).

The pharmacology suggests that the fatty acid presumably released after phospholipase A2 activation is probably oxidized by lipoxygenase, since nordihydroguaiaretic acid, a lipoxygenase inhibitor (Hamberg, 1976; Goetzl et al., 1980), and ETYA, an inhibitor of lipoxygenase and cyclooxygenase (Flower, 1974), inhibited the ATP-, thrombin- and trypsin-induced relaxations, but not those to sodium nitroprusside. Cyclooxygenase does not appear to be involved in the relaxation mechanism, since an inhibitor of cyclooxygenase, indomethacin (Flower, 1974), in fact, potentiated the relaxation to these agents. The potentiation may be due to a nonspecific effect of indomethacin, since, under these conditions (see Methods), relaxations to sodium nitroprusside and isoproterenol were also increased. This may be due to the observation that contractions to norepinephrine were significantly inhibited by indomethacin.

The above results with the various inhibitors are consistent with those of Furchgott (1981), who observed that ATP-induced relaxations were inhibited by ETYA and quinacrine in rabbit aorta. It is not clear why ATP- and thrombin-induced relaxations were not inhibited by ETYA and quinacrine in the canine femoral artery (DeMey and Vanhoutte, 1982). This apparent discrepancy may be due to differences between species and/or vascular tissues.

Although the present results are consistent with the hypothesis that a fatty acid is involved in the chain of events which eventually results in endothelium-dependent relaxation (Furchgott and Zawadzki, 1980a; Furchgott, 1981; Furchgott et al., 1981), it should be noted that the specificity of these various inhibitors has not been established. Many of these agents have also been shown to be ineffective as inhibitors of endothelium-dependent vasodilators in a variety of tissues (for reviews, see Furchgott, 1983; and Rapoport and Murad, 1983b, and references therein). Other agents which have been reported to inhibit the lipoxygenase pathway did not effect relaxation, whereas still other agents not known to inhibit lipoxygenase inhibited the relaxation. Thus, the nature of the endothelium-dependent relaxing factor remains to be established.

The time course of ATP-induced increased levels of cGMP and the onset and development of relaxation were coincident; however, while relaxation was maintained, cGMP levels declined. We have reported similar results with the endothelium-dependent vasodilators, acetylcholine, histamine, and Ca++ ionophore A23187, as well as with sodium nitroprusside (Rapoprt et al., 1983b; Rapoport and Murad, 1983a). Whereas several explanations of this apparent discrepancy may be possible, we suspect that some processes following the formation of cGMP may have a slower turnover rate than cGMP. Indeed, we have shown that the protein phosphorylation profile induced by sodium nitroprusside, which was mimicked by 8-bromo cGMP (Rapoport et al., 1982), acetylcholine, and Ca++ ionophore A23187 (Rapoport et al., 1983a), was similar at times when cGMP levels were elevated and had declined (Rapoport et al., 1983b).

cAMP levels were also elevated by ATP in endothelium- and concentration-dependent manners, although significant elevations occurred after the onset of relaxation. Thus, it may be that ATP induces relaxation through two separate and distinct cyclic nucleotide-dependent mechanisms. The mechanism by which ATP elevates cAMP does not appear to be due to ATP-induced formation of prostacyclin by the endothelial cells (Boeynaems and Galand, 1983; Pearson et al., 1983), and subsequent elevation of cAMP within the smooth muscle due to prostacyclin (Kukovetz et al., 1979; Dembinska-Kiec et al., 1980; Holzmann et al., 1980), since cAMP levels were unaltered by the cyclooxygenase inhibitor, indomethacin (Flower, 1974). Indomethacin has been shown to inhibit adenosine di- and triphosphate-induced formation of prostacyclin from endothelial cells (Boeynaems and Galand, 1983). Another possible explanation is that the endothelium metabolizes ATP to adenosine diphosphate, adenosine monophosphate, and adenosine (Pearson and Gordon, 1979; Pearson et al., 1980), and adenosine acts on the smooth muscle cells to increase cAMP levels (Kukovetz et al., 1978; Goldman et al., 1983). How-
ever, others (Herlihy et al., 1976; Verhaege, 1977) have been unable to demonstrate elevated cAMP levels due to adenosine. Thus, the mechanism by which ATP elevates cAMP remains to be investigated.

The relaxation induced by ATP consisted of both endothelium-dependent and -independent components. Similar results were observed in rabbit thoracic aorta (Furchgott and Zawadzki, 1980b) and, at high ATP concentrations, in canine femoral artery (DeMey and Vanhoutte, 1981).

The mechanism by which the serine proteases, thrombin and trypsin, presumably activate phospholipase $A_2$ is not known. The activation appears to be dependent upon proteolytic activity, since inactivation of thrombin with antithrombin III or hirudin, which inhibit proteolysis (Markwardt, 1961; Joyner et al., 1973; Harpel and Rosenberg, 1976; Griffith, 1982), and trypsin with soybean trypsin inhibitor, inhibit the relaxation. Inactivation of thrombin by antithrombin III and/or hirudin has been reported by others (Joyner et al., 1973; Verhaeghe and Janssens, 1981). In addition, inactivation of thrombin with the proteolytic inhibitor diisopropyl fluorophosphate inhibited thrombin-induced vasodilation (Delin et al., 1967), whereas the binding of thrombin to endothelial cells was unaffected by diisopropyl fluorophosphate pretreatment (Awbrey et al., 1979).

The transient nature of the relaxant effects of the proteases, which has been observed previously for thrombin (Ku, 1982), may be due to proteolytic-induced desensitization, which occurs during exposure to thrombin or trypsin. In support of this hypothesis is the observation that prior exposure to thrombin-induced desensitization to thrombin or trypsin and vice versa. Desensitization to thrombin was accompanied by an inability of thrombin to elevate cGMP levels. Thrombin-induced desensitization as a result of prior thrombin exposure has been observed by others (DeMey et al., 1982). The desensitization does not appear to be due to depletion of arachidonic acid, since incubation with arachidonic acid did not reverse the desensitization (DeMey et al., 1982, and unpublished observation).

The mechanism by which the proteases activate phospholipase $A_2$ appears to be different from that of ATP, since ATP- and acetylcholine-induced relaxations and/or elevated cGMP levels were unaffected by prior exposure to trypsin. Acetylcholine-induced endothelium-dependent relaxation in canine femoral artery was also unaffected by prior thrombin exposure (DeMey et al., 1982).

The pattern of protein phosphorylation induced by ATP, thrombin, and trypsin was identical to that induced by acetylcholine, sodium nitroprusside, and 8-bromo cGMP (Rapoport et al., 1982, 1983a). We have previously shown that the protein phosphorylation profile induced by acetylcholine was abolished in tissues without endothelium (Rapoport et al., 1983a), whereas the profiles with sodium nitroprusside and 8-bromo cGMP were the same in tissues with and without endothelium (Rapoport et al., 1982, 1983a). Furthermore, cGMP-dependent protein kinase activity was increased by acetylcholine and sodium nitroprusside in endothelium-dependent and -independent manners, respectively, in rat thoracic aorta (Fiscus et al., in press). These results suggest that the same functional pool of cGMP is elevated within the smooth muscle through the interaction of acetylcholine, histamine, Ca$^{2+}$ ionophore A23187, ATP, thrombin, or trypsin with the endothelium, as through the direct activation of guanylate cyclase within the smooth muscle by sodium nitroprusside.

It should be noted that a cause-effect relationship between relaxation and cGMP, cGMP-dependent protein kinase, and protein phosphorylation has not been established. However, myosin light chain was dephosphorylated by all the endothelium-dependent and -independent relaxants used in the present and previous studies (Rapoport et al., 1982; 1983a; Draznin et al., 1983). These results suggest that relaxation induced by cGMP may, at least in part, be due to myosin light chain dephosphorylation. The mechanism by which myosin light chain is dephosphorylated may be through alterations in activity of myosin light chain kinase and/or phosphatase; however, this remains to be investigated.

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INDEX TERMS: Vascular smooth muscle • Endothelium • Re-
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