Pharmacologic Receptor Activity of Rabbit Aorta

EFFECT OF DITHIOTHREITOL AND N-ETHYLMALEIMIDE

By Jerome H. Fleisch, Marija Č. Kržan, and Elwood Titus

ABSTRACT

N-ethylmaleimide (NEM), an alkylating agent that covalently binds sulfhydryl groups, and dithiothreitol (DTT, Cleland’s reagent), which reduces disulfide bridges to sulfhydryl groups, affected drug-induced contractions of rabbit aorta. NEM depressed aortic responses to potassium chloride (KCl), norepinephrine, serotonin, histamine, and angiotensin II. This action was attributed to an interaction of NEM with sulfhydryl groups vital to either the membrane function or the contractile apparatus. DTT had minimal effects on responses to KCl, norepinephrine, and serotonin; it potentiated aortic contractions produced by histamine and abolished responses to angiotensin II. Enhancement of the histamine response by DTT was not accompanied by an effect on the histamine-metabolizing enzymes or by an alteration in the $K_B$ and $pA_2$ values for pyrilamine. The results suggest that a disulfide bridge plays a prominent role in both histamine and angiotensin receptor activity of rabbit aorta. With regard to the histamine receptor system, two possibilities exist: (1) reduction of a disulfide bridge at some vital point increases receptor activity or (2) the histamine receptor system, normally in a reduced state, is oxidized in the artificial environment of the tissue bath and needs to be reduced to regain full activity.

KEY WORDS histamine angiotensin norepinephrine serotonin sulfhydryl group disulfide bridge vascular smooth muscle drug receptors

Drug receptor systems can be modified in situ by various procedures including changes in temperature or pH, exposure to enzymes, lipid solvents, protein denaturants, or chelating agents, and chemical reaction with site-directed reagents (1). As these procedures become more selective for one or another receptor, it becomes increasingly likely that an altered macromolecule can be identified and associated with receptor function. Especially with the site-directed drugs, greater selectivity gives greater insight into the nature of a particular receptor system. Among the more group-specific reagents are those which cause oxidation of sulfhydryl groups or reduction of disulfide bridges. Sulfhydryl groups are involved in the oxytocin and vasopressin (2, 3), acetylcholine (4, 5), and alpha receptors (6) of various tissues. The present study was therefore designed to determine whether either of these molecular groupings plays a primary role in drug receptor activity of vascular smooth muscle.

The results showed that N-ethylmaleimide, an agent that covalently binds sulfhydryl groups (7), nonspecifically depressed aortic reactivity. However, dithiothreitol, an agent that reduces disulfide linkages to sulfhydryl groups (8), marked potentiated responses of rabbit aorta to histamine and abolished responses to angiotensin without greatly affecting responses to potassium chloride (KCl), norepinephrine, or serotonin. This phenomenon indicates that the histamine and angiotensin receptor systems of rabbit aorta contain disulfide bridges in prominent positions either in the receptor macromolecule per se or in the chain of events that connect the receptor with the contractile mechanism.

Methods

Male New Zealand rabbits weighing approximately 2 kg were killed either by decapitation or an air embolism. Helically cut thoracic aortic strips were prepared by the method of Puchgott and Bhadrakom (9). Aortas were divided equally into four strips and suspended in 10-ml isolated organ baths containing a modified Krebs-bicarbonate solution (pH 7.4) with the...
following millimolar composition: KCl 4.5, CaCl₂ · 2H₂O 2.5, KH₂PO₄ 1.2, MgSO₄ · 7H₂O 1.2, NaCl 118.2, NaHCO₃ 24.8, and dextrose 10.0.

The aortas were oxygenated with 95% O₂-5% CO₂, and temperature was maintained at 37.5°C with a constant-temperature circulating unit. Tissues were subjected to an initial tension of 2 g. Contractions were measured isometrically with a Grass FT03 force-displacement transducer and recorded on a Grass polygraph as changes in grams of tension. Each aortic strip was kept in the organ bath for 1-2 hours before drugs were tested. All drugs were prepared for each experiment in Krebs-bicarbonate solution. Agonists, except for KCl, were kept on ice throughout the experiment and added to the bath in cumulative doses until a complete dose-response curve was obtained. The bath fluid was exchanged several times after each dose-response curve. Only one agonist and one concentration of antagonist were used per tissue unless otherwise specified. To minimize desensitization that usually occurs between the first and second dose-response curves, three curves were obtained. The second curve served as a control, and the third curve served as the experimental dose-response curve. Between the second and third curves the drug under study was added to the bath. Antagonists were kept in contact with the tissues for at least the following times before drug-induced responses were obtained: phentolamine and pyrilamine were in contact with the tissue for 1 hour and NEM in various concentrations was in contact with the tissue for 15 minutes and then thoroughly washed out during the following 15-20 minutes. To maximize the effects of DTT on aortic receptor systems, a high concentration of DTT was used so that the tissue was exposed to varying concentrations for a standard 15-minute interval and was then thoroughly washed by exchanging the bath fluid several times. After this procedure, the reaction was essentially irreversible. The maximal response to KCl was decreased at concentrations of 3 × 10⁻⁴M and 1 × 10⁻⁴M NEM and all responses were completely abolished after 3 × 10⁻⁴M NEM (Fig. 1, right). Similarly, NEM altered the response to histamine. But unlike its effects on the KCl-induced response, NEM not only decreased the maximal response to histamine but also caused a parallel displacement of the dose-response curve (Fig. 2, bottom). Likewise, NEM antagonized responses of rabbit aorta to serotonin and norepinephrine (Table 1), displaying the same features as those described for the antagonism of histamine. Finally, in one experiment, NEM depressed aortic responses to angiotensin II in a similar manner.

**Results**

**EFFECT OF N-ETHYLMALEIMIDE ON DRUG-INDUCED CONTRACTILE RESPONSES OF RABBIT AORTA**

Since NEM covalently binds sulfhydryl groups, the tissue was exposed to varying concentrations for a standard 15-minute interval and was then thoroughly washed by exchanging the bath fluid several times. After this procedure, the reaction was essentially irreversible. The maximal response to KCl was decreased at concentrations of 3 × 10⁻⁴M and 1 × 10⁻⁴M NEM and all responses were completely abolished after 3 × 10⁻⁴M NEM (Fig. 1, right). Similarly, NEM altered the response to histamine. But unlike its effects on the KCl-induced response, NEM not only decreased the maximal response to histamine but also caused a parallel displacement of the dose-response curve (Fig. 2, bottom). Likewise, NEM antagonized responses of rabbit aorta to serotonin and norepinephrine (Table 1), displaying the same features as those described for the antagonism of histamine. Finally, in one experiment, NEM depressed aortic responses to angiotensin II in a similar manner.

**EFFECTS OF DITHIOTHREITOL ON DRUG-INDUCED CONTRACTILE RESPONSES OF RABBIT AORTA**

The reaction with DTT, unlike that with NEM, is reversible. The drug must remain in contact with the tissue for the duration of the experiment to

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1Both determinations were kindly made by Dr. Michael A. Beaven of the Experimental Therapeutics Branch, the National Heart and Lung Institute.
maintain the sulfhydryl groups in a reduced state. Preliminary experiments indicated that the tissue could tolerate concentrations up to at least $1 \times 10^{-3} M$ for periods of 2–3 hours without any deleterious effects. Experimental dose-response curves were therefore determined after the aorta was exposed to $1 \times 10^{-3} M$ DTT for 1 hour. Figure 1 (left) shows that DTT had very little effect on the response to KCl; the ED$_{50}$ was displaced only slightly, and the maximal response was reduced by 14%. DTT also had very little effect on norepinephrine- and serotonin-induced contractions (Table 2); both dose-response curves were shifted to the right and the values of ED$_{50}$ were increased by 4.5. In contrast to its effect on aortic responses to KCl, norepinephrine, and serotonin, DTT markedly potentiated the response to histamine. In the presence of DTT, there was a shift to the left of the histamine dose-response curve (Fig. 2, top) with the ED$_{50}$ decreasing from $3.8 \times 10^{-7} M$ to $4.0 \times 10^{-7} M$. Of all the agonists tested, angiotensin proved to be the most susceptible to the actions of DTT (Fig. 3); $1 \times 10^{-3} M$ DTT for 1 hour completely abolished the response of the rabbit aorta to angiotensin. Although angiotensin tachyphylaxis has been reported not to be a factor with this tissue (14), we nevertheless took precautions to prevent even slight tachyphylaxis from complicating this finding. Two segments from the same aorta were set up in identical baths only one of which contained $1 \times 10^{-3} M$ DTT, and only one angioten-

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Experimental conditions</th>
<th>ED$_{50}$</th>
<th>% Maximal response ± SE</th>
<th>Number of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>Control</td>
<td>$7.2 \times 10^{-5} M$</td>
<td>100</td>
<td>12</td>
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<tr>
<td></td>
<td>$3 \times 10^{-5} M$ NEM</td>
<td>$1.4 \times 10^{-7} M$</td>
<td>91.7 ± 3.2</td>
<td>5</td>
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<tr>
<td></td>
<td>$1 \times 10^{-4} M$ NEM</td>
<td>$3.4 \times 10^{-8} M$</td>
<td>38.5 ± 2.3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$3 \times 10^{-4} M$ NEM</td>
<td>$3.0 \times 10^{-7} M$</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Control</td>
<td>$3.9 \times 10^{-7} M$</td>
<td>100</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>$3 \times 10^{-5} M$ NEM</td>
<td>$5.0 \times 10^{-7} M$</td>
<td>90.8 ± 3.0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-4} M$ NEM</td>
<td>$3.8 \times 10^{-7} M$</td>
<td>38.5 ± 11.9</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>$3 \times 10^{-4} M$ NEM</td>
<td>$3.0 \times 10^{-7} M$</td>
<td>100</td>
<td>2</td>
</tr>
</tbody>
</table>

* Tissue was exposed to NEM for 15 minutes then thoroughly washed for 15–20 minutes.
† The increase in agor at ED$_{50}$ is expressed as a comparison to control ED$_{50}$ regardless of new maximal response.
‡ $3 \times 10^{-5} M$ NEM abolished all tissue responses.
§ Maximal response was below control ED$_{50}$ with $1 \times 10^{-4} M$ NEM.
TABLE 2

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Experimental conditions</th>
<th>ED₅₀</th>
<th>% Maximal response</th>
<th>Number of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>Control</td>
<td>6.0 × 10⁻⁴M</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁻³M DTT</td>
<td>2.7 × 10⁻⁵M</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Control</td>
<td>3.8 × 10⁻⁴M</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁻³M DTT</td>
<td>1.6 × 10⁻⁴M</td>
<td>103</td>
<td>4</td>
</tr>
</tbody>
</table>

*After control dose-response curves, the tissues were exposed to 1 × 10⁻³M DTT for 1 hour following which dose-response curves were obtained in the presence of the DTT.

HISTAMINASE AND HISTAMINE N-METHYLTRANSFERASE ACTIVITY IN RABBIT AORTA

Rabbit aorta was assayed for histaminase and histamine N-methyltransferase activity to determine if inhibition of histamine-metabolizing enzymes was responsible for the potentiation of the response to histamine by DTT. Histaminase activity was essentially negligible; aortas contained 8–15 units/g in comparison with rat ileum which had over 2500 units/g. There was approximately 25 units/g of histamine N-methyltransferase in aortic tissue compared with around 100 units/g in guinea pig brain. But the histamine N-methyltransferase activity was inhibited only 10% by 1 × 10⁻³M DTT.

EFFECT OF DITHIOTHREITOL ON THE APPARENT DISSOCIATION CONSTANT (Kₘ) AND pA₂ OF PYRILAMINE AND PHENTOLAMINE

The potentiation by DTT of the aortic response to histamine is exhibited as a parallel leftward displacement of the concentration-response curve. Such a displacement could result from an increase in the affinity of the histamine receptor for its agonist. Competitive receptor blocking agents such as pyrilamine (H₁ receptor) and phentolamine (alpha receptor) theoretically compete with their respective agonists for a common site on the receptor macromolecule. If so, then a change in the affinity of the receptor for the agonist might well be reflected in an alteration of the Kᵢ and pA₂ values for the antagonist. Table 3 indicates that no such change occurred; Kᵢ and pA₂ values of control preparations were identical to those of preparations treated with DTT. Furthermore, all slopes of the plots of log(dose ratio — 1) vs. —log[antagonist] were close to the theoretical value of —1 (Table 3) (11), indicating that the agonists were competitive with one another in the presence and the absence of DTT. The experiments with phentolamine, unlike those with pyrilamine, were complicated by the prolonged exposure times that were required for antagonists to achieve their full effects. Thus the usual procedure of incubating the tissue with DTT, followed by the addition of the antagonist with DTT still in the bath, resulted in a preparation that was relatively insensitive to norepinephrine. To circumvent this difficulty, only one dose-response curve was obtained per tissue. One segment of aorta represented the control and the other three segments were exposed to 1 × 10⁻³M DTT for 1 hour; after the first 5 minutes, the three concentrations of phentolamine were added to the baths for the remaining 55 minutes. This procedure was valid as indicated by the Kᵢ, pA₂, and slope obtained (Table 3).

EFFECT OF OXIDIZED DITHIOTHREITOL, ERYTHRITOL, CYSTINE, AND CYSTEINE ON PHARMACOLOGIC RECEPTOR ACTIVITY OF RABBIT AORTA

To rule out the possibility that the potentiation of the response to histamine and the abolition of the response to angiotensin might merely reflect the presence of organic substances in the medium rather than an action of DTT on disulfide bridges, two additional experiments were performed: one used erythritol, the optical isomer of threitol, and the other used oxidized DTT, the cyclic compound formed by the reaction of DTT with disulfide bridges.
bridges. At concentrations up to 0.1M, erythritol had no effect on responses to the two agonists. Oxidized DTT (1 \times 10^{-6}M) for 1 hour also had no effect on the responses to histamine and angiotensin. A few additional experiments with \textit{d,L}-cystine and \textit{l}-cysteine were performed to examine the influence of other agents known to effect sulfur-containing molecular groupings: at 1 \times 10^{-6}M (the highest concentration their solubility would permit) these drugs produced negligible effects on aortic responsiveness to KCl, norepinephrine, serotonin, and histamine. No experiments were performed to determine the effect of cystine and cysteine on the response to angiotensin.

### Discussion

Previous studies have indicated that drug receptor activity could be modified by agents that interact with either disulfide bridges or sulfhydryl groups (1-6). Interestingly, thiols (compounds containing a free sulfhydryl group) have been shown to both antagonize (3) and potentiate (15, 16) pharmacologic receptor activity in isolated smooth muscle preparations. The latter studies involved the potentiation of responses to bradykinin by cysteine, a monothiol. Although initial experiments suggested that inhibition of kininase was responsible for the increased sensitivity of guinea pig ileum to bradykinin, later investigations cast doubt on this hypothesis (17, 18). Recently, Potter and Walaszek (19) concluded that cysteine potentiates responses to bradykinin by facilitating bradykinin-induced release of acetylcholine from nerve endings. The aorta is more satisfactory than intestinal smooth muscle for such study. It is sparsely innervated, and release of neurohormone by an agonist is not as prominent as it is with ileum (20, 21).

In the present study the actions of NEM and DTT on aortic smooth muscle membrane and contractile apparatus were ascertained by examining the effects of these agents on responses to KCl. KCl contracts smooth muscle by depolarizing the membrane; this process is not a conventional drug receptor mechanism (22). NEM depressed aortic responses to KCl, norepinephrine, serotonin, histamine, and angiotensin in a manner similar to other alkylating agents (i.e., Dibenamine) (23, 24) and the local anesthetics (25). These results are attributed to the interaction of NEM with a sulfhydryl group vital to either membrane function or the contractile apparatus. This apparent nonspecificity therefore precluded the use of this agent in discerning the role of sulfhydryl groups in drug receptor activity.

DTT proved to be more useful than NEM. To maximize the influence of this disulfide bridge reducing agent on drug receptor activity, rabbit aorta was exposed to a relatively high concentration (1 \times 10^{-6}M) of DTT for 1 hour. DTT had only a meager effect on responses to KCl, norepinephrine, serotonin, and histamine. The maximal response to KCl was slightly reduced and the values of ED$_{50}$ for norepinephrine and serotonin were increased approximately 4.5-fold. DTT greatly potentiated aortic contractions produced by histamine. This potentiation was characterized by a 10-fold decrease in the ED$_{50}$ with no change in the maximal response. It thus appeared that DTT reduced a disulfide linkage at some essential location in the histamine receptor.

The next series of experiments was designed to determine if DTT inhibited enzymes responsible for the degradation of histamine. If this effect occurred, then the potentiation would be similar to that seen with acetylcholine after cholinesterase inhibition. Determination of histaminase and histamine N-methyltransferase revealed low levels of these enzymes in aortic tissue. Furthermore, 1 \times 10^{-3}M DTT only slightly inhibited histamine N-methyltransferase. Inhibition of these enzymes was therefore discounted as being responsible for the potentiation of the response to histamine by DTT.

### Table 3

<table>
<thead>
<tr>
<th>Agonist/antagonist</th>
<th>Experimental conditions</th>
<th>$K_a$ ± se*</th>
<th>$pA_2$</th>
<th>Slope†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine/pyrilamine</td>
<td>Control</td>
<td>2.9 \times 10^{-9} \pm 0.3 \times 10^{-9}</td>
<td>8.78</td>
<td>-0.88</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-6}M$ DTT</td>
<td>2.3 \times 10^{-9} \pm 0.4 \times 10^{-9}</td>
<td>8.71</td>
<td>-0.97</td>
</tr>
<tr>
<td>Norepinephrine/phen tolamine</td>
<td>Control</td>
<td>1.6 \times 10^{-9} \pm 0.1 \times 10^{-9}</td>
<td>7.79</td>
<td>-1.00</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-6}M$ DTT</td>
<td>2.1 \times 10^{-9} \pm 0.4 \times 10^{-9}</td>
<td>7.76</td>
<td>-0.98</td>
</tr>
</tbody>
</table>

*Each value is the mean ± se of four experiments.
†Slope of the plot of log(dose ratio - 1) vs. - log [antagonist].

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\[7.76\] \[7.79\] \[8.71\] \[8.78\]
An increase in the affinity constant of the histamine receptor would also result in an increased tissue responsiveness to histamine. It might be expected that such a change would be accompanied by a concomitant change in the apparent dissociation constant of the receptor-inhibitor complex \( K_B \), since Fleisch and Titus (25) showed that local anesthetics, which produce a parallel displacement of acetylcholine and norepinephrine dose-response curves, also alter the \( K_B \) and \( pA_2 \) values for atropine and phentolamine. There was no influence by DTT on \( K_B \) and \( pA_2 \) values for pyrilamine (\( H_1 \)-receptor blocking agent) in aortic tissue. Furthermore, \( K_B \) and \( pA_2 \) values for phentolamine (alpha-receptor blocking agent) were identical in the presence and the absence of DTT even though the norepinephrine \( E_D_{50} \) was increased 4.5-fold. If pyrilamine and phentolamine compete with histamine and norepinephrine, respectively, for the same receptor sites, then we can conclude that DTT did not modify the receptor site per se unless, of course, \( K_B \) and \( pA_2 \) values are not sensitive indicators in this situation. An alternative explanation that is the most compatible with the evidence obtained at this point in the investigation would be that DTT reduced a disulfide bridge at one of the steps in the sequence of events that connects the receptor with the contractile mechanism.

Since bradykinin, a polypeptide that has been previously shown to be potentiated by thiols (15, 16), only contracts rabbit aorta slightly, if at all, the effects of DTT on another polypeptide, angiotensin II, were investigated. DTT abolished contractions produced by the octapeptide. Concentrations up to 1000 times that producing the control \( E_D_{50} \) were inactive.

To substantiate our view that the actions of DTT were due to disulfide bridge reduction and not to another property of the molecule, the effects of erythritol and oxidized DTT were examined on aortic responses to histamine and angiotensin. Erythritol (up to 0.1M) and oxidized DTT (1 \( \times 10^{-3} \)M) were without effect on contractions induced by histamine and angiotensin. Unlike DTT, cysteine, another disulfide bridge reducing agent, was without effect on responses to KCl, norepinephrine, serotonin, and histamine. This phenomenon may have been related to the difference in their redox potentials (that of DTT is more negative than that of cysteine, and therefore DTT is a better reducing agent [8]). Also, the solubility of cysteine precluded the use of higher and possibly more effective concentrations.

The present study suggests that a disulfide bridge plays a prominent role in angiotensin receptor activity of rabbit aorta. Reduction of disulfide bridges to sulfhydryl groups results in inactivation of the response to angiotensin. With regard to the histamine receptor system, two possibilities exist: (1) reduction of a disulfide bridge at some vital point increases receptor activity or (2) the histamine receptor system, normally in a reduced state, is oxidized in the artificial environment of the tissue bath and needs to be reduced to regain full activity.

References


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