ABSTRACT

The anticholinesterase agents, physostigmine, neostigmine, and diisopro-
pylfluorophosphonate were studied for their effect on the vasoconstrictor responses of perfused mesenteric arteries of rat produced by stimulation of their sympathetic postganglionic nerves. The experiments were performed at 30°C. All three anticholinesterase drugs greatly potentiated the vasoconstrictor response to nerve stimulation at a frequency of 1/sec, increased it less at a frequency of 2/sec, and still less at a frequency of 3/sec. At a frequency of 6/sec, there was practically no increase. The infusion of physostigmine potentiated slightly the response to injected norepinephrine, whereas the infusion of neostigmine or diisopropylfluorophosphonate did not alter the response to injected norepinephrine. The increase in response to adrenergic nerve stimulation produced by anticholinesterase agents was interpreted to be due to the inactivation of cholinesterase, thereby causing an increased accumulation of acetylcholine which in turn liberated more norepinephrine from the sympathetic postganglionic fibers.

ADDITIONAL KEY WORDS sympathetic mechanism in mesenteric arteries cholinesterase activity cholinergic link hypothesis neostigmine physostigmine augmentation of perfusion pressure responses diisopropylfluorophosphonate

In an earlier paper (1), experiments were described in which the rat superior mesenteric artery and its branches were perfused with Tyrode's solution according to the method of McGregor (2). Stimulation of the sympathetic postganglionic fibers supplying the mesenteric vasculature produced vasoconstriction as evidenced by a rise of pressure in the cannula through which the perfusion fluid was delivered at a constant rate. When acetylcholine was added to the perfusion fluid, the vasoconstriction produced by nerve stimulation was modified in different ways depending on the dose. Thus, acetylcholine at very low concentrations, .05 ng/ml, increased the constrictor response during 30 minutes of infusion; it also increased the constrictor response at higher concentration, 2 ng/ml, when infused for 15 seconds. When, however, the same concentration or any higher concentration of acetylcholine was infused for 10 minutes or more, the constrictor response produced by stimulation was greatly reduced. Acetylcholine at a concentration of 50 ng/ml often abolished it. These observations indicate that the stimulation of the postganglionic fibers releases acetylcholine which acts on receptors at the nerve terminal to release norepinephrine. The infusion of very low concentrations of acetylcholine adds its effect to that of acetylcholine released by the nerve impulse, and in this way a greater release of norepinephrine occurs. However, when more acetylcholine was infused, it occupied all the receptors at the nerve ending containing norepinephrine so that acetylcholine released by the nerve impulse was without effect.

To test this view, experiments were done with...
the anticholinesterase agents, physostigmine, neostigmine, and diisopropylfluorophosphonate (DFP), to determine if they augmented the vasoconstrictor response of mesenteric arteries to stimulation of the sympathetic nerves. Since it has been suggested (3) that an anticholinesterase agent would show the greatest effect at low frequencies of stimulation, the experiments have been carried out at frequencies of 1, 2, 3, and 6/sec. The effect of the infusion of physostigmine, neostigmine, and DFP on the response to injected norepinephrine was also determined. Anticholinesterase agents were found to increase considerably the response to nerve stimulation at the lowest frequencies.

Methods

Female albino rats, weighing 250 to 300 g, were used. The superior mesenteric artery with its small resistance vessels was isolated and cannulated as described by McGregor (3). The vessels were perfused with Tyrode’s solution at a constant rate of 25 ml/min with a Harvard peristaltic pump (Model 1210) as described earlier (1). The temperature of the perfusion fluid was usually 30°C; some preparations were perfused at 22°C. The solution was aerated with 95% O₂ and 5% CO₂. The pressure in the cannula perfusing the artery was recorded on a kymograph with a frontal writing lever. Before cannulation of the superior mesenteric artery, the pressure was 60 mm Hg. During perfusion of the arteries, the pressure was 80 mm Hg at 30°C. Thus, the basal arterial pressure in these experiments was 60 mm Hg at 22°C. Since the mesenteric vessels were cut where they entered the intestine, this basal pressure was maintained by the resistance of arterioles. As the pump maintained a constant flow of 25 ml/min, increase in the perfusion pressure represented narrowing of the arterioles.

A bipolar platinum electrode was placed on the artery with its accompanying nerves about 5 mm distal to the cannula. The perivascular sympathetic nerves were stimulated for periods of 20 to 40 seconds at 4-minute intervals with a Grass stimulator (Model S4) using biphasic rectangular pulses (20 v, 1 msec) at different frequencies. Norepinephrine was injected directly into the cannula leading to the superior mesenteric artery. All other drugs were added to Tyrode’s solution in a reservoir identical to that containing the control solution. The perfusion solutions were changed by simultaneously opening that from the other.

Drugs—Norepinephrine (L-noreadrenaline bitartrate monohydrate) and diisopropylfluorophosphonate (DFP) were obtained from K & K Laboratories, physostigmine (eserine sulfate) from Nutritional Biochemicals Corporation, and neostigmine (prostigmin sulfate) was generously provided by CIBA (Basel).

All drugs except DFP were dissolved just before use in small volumes of Tyrode’s solution so that when 0.2 ml was added to 1 liter of the perfusion fluid the required concentration for perfusion was obtained. DFP was dissolved in propylene glycol and 0.2 ml of this solution added to 1 liter of Tyrode’s solution to obtain the final concentration.

Results

EFFECTS OF THE INFUSION OF PHYSOSTIGMINE, NEOSTIGMINE, AND DFP ON THE RESPONSES TO SYMPATHETIC NERVE STIMULATION AND INJECTED NOREPINEPHRINE

When physostigmine, neostigmine, or DFP was infused, the vasoconstriction produced by nerve stimulation was greatly increased at lower frequencies of stimulation. The experiments were performed at frequencies of 1, 2, 3, and 6/sec since these frequencies are within physiological limits (4). In each experiment a fixed number of shocks was given. The response was measured as the rise in perfusion pressure produced by nerve stimulation. In each experiment at least four to six control responses were recorded before the infusion of an anticholinesterase agent. The increase in response to nerve stimulation produced by the infusion of an anticholinesterase agent was calculated by measuring the height of the maximum response obtained during that period and expressing it as a percent of the initial control response.

When physostigmine was infused in concentrations of 2 µg/ml, the mean increase in response to nerve stimulation at 1/sec was 62% (two experiments), when the concentration was 3 µg/ml, the mean increase was 155% (two experiments), and when the concentration was 6 µg/ml, the mean increase was 205% (two experiments). The concentration of physostigmine was kept at 6 µg/ml in the remaining experiments. The concentration of neostigmine was 2 µg/ml and of DFP, 2 µg/ml.

The infusion of each of the anticholinester-
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649

TOO

500

400

300

200

100

FREQUENCY (PER SECOND) OF NERVE STIMULATION

FIGURE 1

Effect of the infusion of physostigmine, neostigmine, and diisopropylfluorophosphonate (DFP) on the response of perfused mesenteric arteries of rat to sympathetic nerve stimulation at different frequencies. The mesenteric arteries were perfused with Tyrode's solution at a rate of 25 ml/min at 30°C. The perivascular nerves were stimulated with biphasic rectangular pulses (20 v, 1 msec) at different frequencies every 4 minutes for 20 to 40 seconds. After recording four to six control responses to stimulation, physostigmine (6 μg/ml), neostigmine (2 μg/ml), or DFP (2 μg/ml) was infused for 20 to 25 minutes and further responses recorded. Circles = effect of physostigmine; crosses = effect of neostigmine; triangles = effect of DFP. Ordinate = mean percent increase in response expressed as percent of the control responses; abscissa = log of frequency per second of nerve stimulation. The number in parentheses indicates number of preparations perfused.

Physostigmine, neostigmine, or DFP was continuously infused over a longer period (more than 25 minutes). Rather, the responses declined while the anticholinesterase agent was still being infused. In two experiments with neostigmine and two with physostigmine at 1/sec, the decline in response continued until the responses to stimulation were less than before their infusion. When the infusion of these agents was stopped, the responses then rose again to their initial control height. When physostigmine or neostigmine was infused for 20 to 25 minutes, the increase in response was usually maintained.
for about an hour after resuming perfusion with drug-free normal Tyrode's solution. The infusion of DFP for 20 to 25 minutes produced an increase in response to stimulation which lasted as long as responses were recorded (2 hours). Figure 2 illustrates an example, where the infusion of DFP produced an increase in response to nerve stimulation at 2/sec.

The potentiation in response to stimulation produced by the infusion of physostigmine, neostigmine, and DFP at 1/sec was often equal to the magnitude of the response recorded at 6/sec in the absence of these agents. Figure 3 illustrates an example where the responses to stimulation were recorded at frequencies of 1, 2, 3, and 6/sec and then the frequency was kept at 1/sec during the whole experiment. The infusion of neostigmine so greatly potentiated the response at 1/sec that the height of the increased response was equal to that recorded at 6/sec without the infusion of neostigmine. The failure of anticholinesterase agents to potentiate greatly the response to stimulation at 6/sec was not due to the height of the initial control response because responses of still higher magnitude could be obtained by increasing the frequency, duration, or strength of the stimulus.

**EFFECT OF THE INFUSION OF ANTICHOLINESTERASE AGENTS ON RESPONSE TO INJECTED NOREPINEPHRINE**

Potentiation of the responses to sympathetic nerve stimulation during infusion of an anticholinesterase agent may have been due to enhanced vasoconstriction produced by norepinephrine released by nerve stimulation. Experiments were carried out to explore this possibility by comparing the responses to submaximal nerve stimulation at 1/sec with those to submaximal amounts of injected norepinephrine in the presence of physostigmine, 6 μg/ml (eight experiments), neostigmine, 2 μg/ml (eight experiments), and DFP, 2 μg/ml (six experiments). The total dose of norepinephrine (100 to 300 ng) injected into the cannula leading to the superior mesenteric artery produced a vasoconstrictor response of the same magnitude as that elicited by nerve stimulation at 1/sec. The results in Figure 4 show that the infusion of physostigmine (6 μg/ml) slightly increased the response to injected norepinephrine, but produced a much greater increase in the response to sympathetic nerve stimulation. Neostigmine and DFP did not alter the

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**FIGURE 2**

*Effect of diisopropylfluorophosphonate (DFP) on the responses to sympathetic nerve stimulation at 2/sec for 20 seconds. The infusion of DFP, 2 μg/ml, augmented the responses greatly; some increase continued on restoration of drug-free Tyrode's solution.*

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**Figure 3**
Effect of neostigmine (Neostig.) on the responses to sympathetic nerve stimulation at 1/sec for 30 seconds. On the left are responses to stimulation recorded at 1, 2, 3, and 6/sec, respectively. All other responses were elicited at 1/sec. The infusion of neostigmine, 2 µg/ml, potentiated the response to stimulation at 1/sec so markedly that the height of the responses was equal to the magnitude of the response recorded at 6/sec in the absence of neostigmine.

**Figure 4**
Comparison of the potentiating effect of physostigmine on the responses of perfused mesenteric arteries of rat to submaximal sympathetic nerve stimulation and to submaximal amounts of injected norepinephrine. Open circles = responses to nerve stimulation (20 v, 1 msec) at 1/sec every 4 minutes for 40 seconds; solid circles = responses to injected norepinephrine (100 ng). The responses to injected norepinephrine were observed by injecting it directly into the cannula leading to the superior mesenteric artery. The infusion of physostigmine, 8 µg/ml, although potentiating the response to injected norepinephrine slightly, produced a much greater increase in response to nerve stimulation.
response to injected norepinephrine either at the beginning or at the end of the infusion, though the infusion of these agents greatly increased the response to nerve stimulation. Figure 5 illustrates this effect of neostigmine.

**EFFECT OF THE INFUSION OF ANTICHOLINESTERASE AGENTS ON THE RESPONSE TO NERVE STIMULATION AT 22°C**

The amplitude of responses to nerve stimulation (1/sec) at 22°C was not different from that at 30°C. Perfusion with Tyrode’s solution at 22°C greatly reduced the potentiation of the response to nerve stimulation produced by anticholinesterase agents. The mean percent increase in response to nerve stimulation at 1/sec produced by their infusion (Table 1) shows a marked difference in the potentiation of response produced by these agents at 22°C and 30°C. The results at 30°C in Table 1 are those taken from Figure 1. The increase in response to nerve stimulation by anticholinesterase agents either at 22°C or at 30°C was found to be independent of the amplitude of initial control responses.

**EFFECT OF PROPYLENE GLYCOL**

Since DFP was dissolved in propylene glycol and then added in Tyrode’s solution, its action on the response to nerve stimulation and injected norepinephrine was also determined. Propylene glycol in concentrations of 0.2 ml/liter of Tyrode’s solution produced no effect either on the response to nerve stimulation or injected norepinephrine.

**Discussion**

The vasoconstrictor response following stimulation of the sympathetic fibers to the branches of the superior mesenteric artery of the rat increased when either physostigmine, neostigmine, or DFP was infused; the increase being greatest at the lowest frequency of stimulation. That the fibers were indeed postganglionic was shown by McGregor (2) who found that the response to stimulation was unaffected by hexamethonium and was blocked by bretylium and guanethidine. These findings have been confirmed (5). Moreover, it has been observed that the ganglion-blocking agent, tetraethylammonium, at concentrations up to 50 μg/ml, enhanced the responses of mesenteric arteries to periartrial nerve stimulation without affecting the responses of equal magnitude to injected norepinephrine.
TABLE 1

Mean Percent Increase in Response of Mesenteric Arteries to Sympathetic Postganglionic Nerve Stimulation at 1/sec Produced by the Infusion of Anticholinesterase Agents at Different Temperatures

<table>
<thead>
<tr>
<th>Anticholinesterase Agent</th>
<th>Temperature (°C)</th>
<th>No. of exptrn.</th>
<th>Mean percent increase in response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physostigmine</td>
<td>22</td>
<td>5</td>
<td>11 ± 9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>10</td>
<td>241 ± 53</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>22</td>
<td>6</td>
<td>16 ± 11</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>10</td>
<td>530 ± 35</td>
</tr>
<tr>
<td>Diisopropylfluorophosphate</td>
<td>22</td>
<td>5</td>
<td>27 ± 17</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>10</td>
<td>386 ± 23</td>
</tr>
</tbody>
</table>

Leach and Zumani (6) have reported that the vasoconstrictor responses of mesenteric arteries to tyramine were absent in rats previously treated with 6-OH dopamine or surgically denervated. That physostigmine, neostigmine, or DFP increased the response to postganglionic stimulation so strikingly cannot be explained if the sympathetic impulse releases norepinephrine directly. Thus the effect of infusion of these agents shows that acetylcholine is involved in the release. This is not unexpected since the postganglionic fiber has the same embryonic origin as the chromaffin cells of the adrenal medulla, where the release of catecholamines by acetylcholine is dependent on the concentration of calcium (7). The release of norepinephrine from the postganglionic fiber either by stimulation of the fiber (8) or by the action of acetylcholine (9) is also dependent on the concentration of calcium.

The results of this study indicated that the response to nerve stimulation was enhanced most at the lowest frequencies by infusion of the anticholinesterase agents. At a frequency of 1/sec, cholinesterase has 1 second in which to destroy the acetylcholine liberated by one shock before the next shock arrives. At a frequency of 2/sec, the cholinesterase has only 0.5 second for this action. The concentration of acetylcholine following a series of shocks at 2/sec will therefore increase at a much slower rate than following a series of shocks at 2/sec. If it is assumed that the amount of norepinephrine released is determined by the concentration of acetylcholine, then the increase in the response to postganglionic stimulation with increase of frequency is explained by the decreased time for cholinesterase action. However, in the presence of physostigmine, neostigmine, or DFP, the inability of cholinesterase to destroy acetylcholine would result in an increased concentration of acetylcholine which acts on the receptors at the terminal containing norepinephrine. Thus a great increase in the response would result when the frequency was lowest. The increase in response to nerve stimulation at 1/sec produced by the infusion of physostigmine, neostigmine, or DFP was usually equal to the magnitude of the responses recorded at 6/sec in the absence of these agents.

The increased response to nerve stimulation was not due to increased sensitivity of vascular smooth muscle to norepinephrine since these agents did not alter the response to injected norepinephrine. Physostigmine was the only agent that produced a small increase in response to injected norepinephrine which was in no way comparable to its much greater effect on response to nerve stimulation. Since the basal pressure during the infusion of physostigmine remained unchanged, it is unlikely that this potentiation is due to a direct action on the smooth muscle of vessels. A satisfactory explanation for the small potentiation in response to injected norepi-
nephine produced by physostigmine is not now available.

The present experiments were performed at 30°C in contrast to earlier experiments with acetylcholine (1) which were carried out at 22°C. The temperature of the perfusion fluid was raised to 30°C when it was found that the anticholinesterase agents had only a slight effect at 22°C. The temperature of 22°C was originally chosen for the experiments with acetylcholine (1) because the response to nerve stimulation remained uniform for 3 to 4 hours. Since at 22°C the amplitude of responses to nerve stimulation at 1/sec was not very different from that at 30°C, and moreover the potentiation in response to nerve stimulation produced by anticholinesterase agents either at 22°C or 30°C was independent of the amplitude of initial control responses, the greater effect of anticholinesterase agents at 30°C may be due to higher activity of cholinesterase of the nerve. It has been shown earlier (10) that the responses to nerve stimulation at 7/sec was greatly reduced and the responses to injected norepinephrine greatly increased at 35°C. The earlier observations (1) also demonstrated that the action of acetylcholine in diminishing or abolishing the response to stimulation was also much less at 37°C and that, although a concentration as low as 2 ng/ml was sufficient to reduce the response greatly at 22°C, a concentration of 8 ng/ml caused no reduction whatever at 37°C. The latter appears to be due to reduced activity of cholinesterase at 22°C.

Evidence that anticholinesterase agents increase the response to stimulation of sympathetic postganglionic fibers has been obtained before, but the effects described in this paper are of greater magnitude than those made previously. Evidence was obtained previously in the nictitating membrane of the cat (11), in the femoral artery of dog (12), in the isolated taenia of the guinea-pig (13), in the isolated rabbit heart (14), and in the retractor penis of the dog (15). In all these tissues, it was shown that the greatest increase was observed at the lowest frequency of stimulation and that the increase diminished as the frequency rose. In the perfused vessels of the rabbit ear (16) and in the renal blood flow in the dog (17), an increase in the response was again observed in the presence of physostigmine, but there was no comparison of the increase at different frequencies. How does acetylcholine affect the release of norepinephrine? The work of Ehinger and his colleagues (18, 19) provides a clue. They described fibers containing norepinephrine running together with fibers containing cholinesterase in the rat iris; they said "there is a morphological foundation for a direct interaction in the peripheral tissues between concomitant cholinergic and adrenergic nerves." Recently acetylcholinesterase and butyrylcholinesterase activities have been demonstrated by the staining technique in the artery of the rabbit's ear at the medial-advential border (20), a region known to contain a noradrenergic nerve terminal network (21). The stains of these enzymes disappear after degeneration of the sympathetic nerves following superior cervical ganglionectomy but not in rabbits treated with reserpine (20). These findings are supported by an electron micrograph published by Tranzer and Thoenen (22) who showed in nerve endings in the cat iris that one profile full of agranular vesicles could be contiguous to a second profile containing dense-core vesicles. These observed suggest that nerve impulses may release acetylcholine from nerve endings containing agranular vesicles. These nerve endings may be those of cholinergic fibers. The acetylcholine released from these fibers acts on some receptors at the adrenergic nerve ending containing dense-core vesicles and makes this ending permeable to calcium ions. These ions enter the ending and release norepinephrine from the dense-core vesicles.

The state of knowledge based on pharmacological evidence concerning release of norepinephrine seems to be fairly complete and at least comparable to similar evidence for release of acetylcholine from motor nerve endings (23). However, in contrast to the release of acetylcholine from motor nerves (24), the exact details of release of norepinephrine from the sympathetic postganglionic

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fibers are presently undefined. An electron microscopic study of adrenergic nerve terminals in the rat heart was published by Elinger et al. (25). The adrenergic nerve terminals in animals treated with 5-hydroxydopamine showed synaptic vesicles containing dense-core granules. These findings were shown to be contiguous to the endings containing agranular vesicles that are cholinergic. These findings support the pharmacological evidence for a "cholinergic link" in the release of the adrenergic transmitter in mesenteric arteries.

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References

Potentiation by Anticholinesterases of the Response of Rat Mesenteric Arteries to Sympathetic Postganglionic Nerve Stimulation

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