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THE EFFECT OF HEMICHOLINIUM (HC-3) ON SYMPATHETIC TRANSMISSION AT THE NICTITATING MEMBRANE OF THE RABBIT

BY

D. JACOBOWITZ, P. JOHNSON, I. KITCHNER AND G. B. KOELLE

From the Department of Pharmacology, Schools of Medicine, University of Pennsylvania, Philadelphia 4, *Pa., U.S.A.*

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The effects of certain drugs on the mechanical and electrical responses of the nictitating membrane of the cat to sympathetic nerve stimulation have been studied extensively in conjunction with the hypothesis of Burn & Rand (1959, 1962) that acetylcholine is involved in the peripheral release of noradrenaline by postganglionic adrenergic fibres. Results with anticholinesterase and cholinergic blocking agents have been variable, probably because of differences in methods, and have been interpreted both as consistent (Burn, Dromey & Large, 1963; Burn, Rand & Wien, 1963; Bowman, Callingham & Cuthbert, 1964) and as inconsistent (Gardiner, Hellmann & Thompson, 1962; Nystrom, 1962) with the hypothesis. On the other hand, hemicholinium (α,α'-dimethylaminoethanol-4,4'-biacetophenone ; HC-3), a compound which interferes with the synthesis of acetylcholine by nerve fibres, was found not to modify the response of the postganglionically stimulated sympathetic nerve-nictitating membrane preparation, either *in vivo* (Wilson & Long, 1959) or *in vitro* (Gardiner & Thompson, 1961). Since transmission at this site is readily prevented by adrenergic blocking agents, these findings were considered as definite evidence against the participation of acetylcholine in adrenergic transmission.

Recent histochemical studies of the autonomic innervation of the smooth muscle of the nictitating membranes of the cat and rabbit have shown that in both species there is a rich supply of catechol amine-containing (adrenergic) fibres. The same region of the rabbit nictitating membrane showed also a similar distribution of acetylcholinesterase-staining fibres, whereas that of the cat contained a considerably smaller number of the latter (Jacobowitz & Koelle, 1965). This difference in acetylcholinesterase-staining seen in the innervation of the nictitating membranes of the two species is similar to that in the acetylcholinesterase contents of their superior cervical ganglion cells observed earlier (Koelle, 1953, 1955). Furthermore, excision of the ganglion resulted in a definite reduction of acetylcholinesterase in the innervation of the smooth muscle of the rabbit membrane, but there was no detectable change in that of the cat; in both species, fluorescence due to catechol amines disappeared completely after the operation. Since the presence of acetylcholinesterase in neurones in general reflects the associated occurrence of acetylcholine and

choline acetylase (for references, see Jacobowitz & Koelle, 1965), these observations suggested that acetylcholine might participate more directly in adrenergic transmission in the nictitating membrane of the rabbit than in that of the cat. Therefore, we have studied the effect of hemicholinium on this preparation in the rabbit, and have included a small number of confirmatory experiments on the cat. An abstract of these findings has been published (Jacobowitz & Koelle, 1964).

METHODS

Fifteen rabbits weighing 3 to 5 kg were used in the main series of experiments; they included both sexes of albino and chinchilla types. The rabbits were anaesthetized with 0.7 ml. of Dial-Urethane per kg body weight, intravenously. The three cats studied weighed between 2.5 and 3.5 kg; they were anaesthetized with 30 mg of sodium pentobarbitone per kg body weight, intraperitoneally. A tracheal cannula was inserted, and artificial ventilation was administered with a Palmer pump when necessary. The superior cervical ganglion was exposed and bipolar electrodes were placed on the ganglion. After the electrodes had been fixed in position they were submerged in mineral oil. The preganglionic sympathetic trunk on the exposed side and the vagus nerves on both sides were tied and cut. The cornea was incised with a scalpel and the lens and vitreous humour were expelled in order to deflate the eye. The eyelids were sewn back and the head was fixed in a clamp as rigidly as possible. Isometric contractions of the nictitating membrane were recorded by connecting it with a silk thread to a pressure transducer leading to a Grass Polygraph. The ganglion was stimulated at a frequency of 20 shocks/sec, with a pulse duration of 0.5 msec and a voltage that would cause a maximal contraction of the nictitating membrane. Stimuli were applied for 10 sec, beginning every 30 sec.

The rabbits were placed in three groups for this series of experiments: a control group (three rabbits), which received no drug; a group (five rabbits) which received 0.05 mg/kg of hemicholinium, into a femoral vein, after approximately 30 min of nerve stimulation; and a group (seven rabbits) which received 0.10 mg/kg of hemicholinium under similar conditions. Rabbits were assigned to the three groups in random sequence and contractions were recorded for 1 hr after the initial 30-min control period. In order to test the assumption that postganglionic neuroeffector transmission at the rabbit nictitating membrane is essentially adrenergic, two additional rabbits were given total cumulative doses of 2.0 and 3.0 mg/kg of phentolamine, intravenously, over periods of 40 and 56 min, respectively; after the maximal effect of the last dose on the response of the nictitating membrane, they received atropine sulphate, 0.5 and 0.66 mg/kg, intravenously, respectively. The three cats were given successive intravenous injections of hemicholinium in doses ranging from 0.05 to 1.0 mg/kg at various intervals; stimulation and recording were performed as with the rabbits.

Results were calculated as follows. The vertical distance from the top of the baseline to the peak recorded during stimulation was measured for the five contractions obtained immediately before the first injection of drug; the mean of this value was taken for the control. At the intervals indicated after the administration of drug, the heights of the succeeding five contractions were measured similarly, and the mean values expressed as percentages of the control response.

The effects of hemicholinium on respiration and blood pressure were measured in four rabbits, using the dosage range employed in the nictitating membrane experiments. Respiration was monitored by a blood pressure cuff wrapped around the thorax, and blood pressure from a polyethylene catheter inserted into a common carotid artery; both were connected with appropriate pressure transducers for amplification and recording on an ink-writing Sanborn polygraph. Test doses of acetylcholine (0.033 to 0.4 μg/kg, intravenously) and noradrenaline (2.0 μg/kg, intravenously) were given before and at intervals after the administration of hemicholinium. In two of these experiments, contractions of the nictitating membranes were also measured, using the same type of stimulation and recording as previously.

RESULTS

The maximal tension of isometric contraction of the rabbit nictitating membrane developed with nerve stimulation during the control periods ranged approximately from

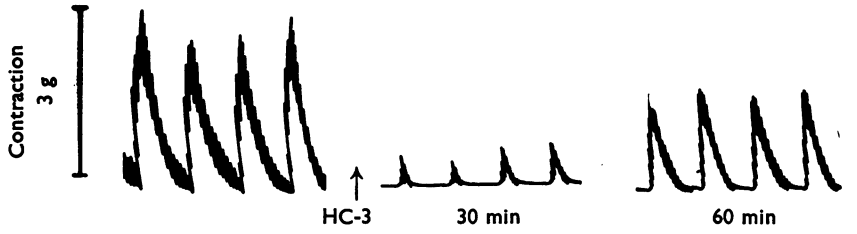

Fig. 1. Effect of hemicholinium (HC-3) on isometric contraction of the rabbit nictitating membrane in response to tetanic stimulation (20 shocks/sec for 10 sec, every 30 sec) of the postganglionic cervical sympathetic trunk. Calibration of contraction tension on the left. Left: control; centre: 30 min after hemicholinium, 0.05 mg/kg, intravenously; right: 60 min after hemicholinium.

1 to 3 g, but was fairly constant for each animal. In spite of the rigid fixation of the head, respiratory excursions were superimposed on the recordings of the membrane contractions.

A portion of a typical result from an experiment on the rabbit nictitating membrane is shown in Fig. 1. At 30 min after the lower dose of hemicholinium tested (0.05 mg/kg), there was a considerable reduction in the response to ganglionic stimulation. At the end of 60 min, the response had returned considerably toward the control height.

The results of all fifteen rabbit experiments in the main series are shown graphically in Fig. 2, where the mean tensions, expressed as percentages of the control tensions, for all the animals of each group are plotted at 10 min intervals for the 60 min experimental period. While there was only a slight fall in the mean responses of the control group over the full period, the contractions of both experimental groups were reduced to approximately

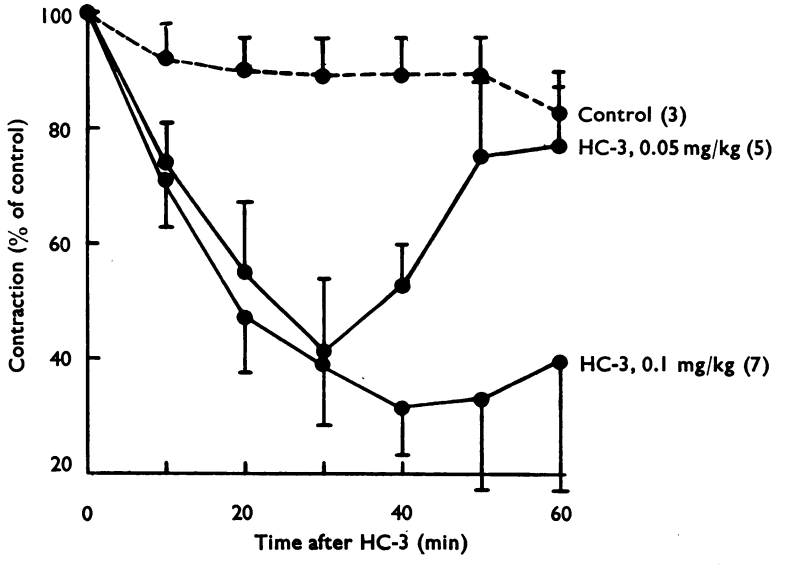

Fig. 2. Effect of hemicholinium (HC-3) on isometric contraction of rabbit nictitating membranes, under same conditions as for Fig. 1. Each point represents the mean response of the number of rabbits shown in parentheses. Vertical lines indicate standard errors of the means.

40% of the initial values at the end of 30 min. Thereafter, the height of contraction began to return in the lower dose group, but fell still lower, and had returned considerably less by the end of 1 hr in the higher dose group. The curves bear a strong resemblance to those published by Wilson & Long (1959, Fig. 3) showing the effects of hemicholinium in a similar dosage range on a preparation involving typically cholinergic transmission, the salivary flow in response to chorda tympani stimulation in the dog.

Pharmacological evidence of the essentially adrenergic nature of the sympathetic innervation of the rabbit nictitating membrane was provided by the results of the studies with phentolamine. In one rabbit, the cumulative dose of 2 mg/kg (1+1 mg/kg) reduced the response to 30% of the control value; atropine sulphate (0.5 mg/kg) caused a further reduction to 15%. In the other, the same total dose of phentolamine (0.6+0.4+1.0 mg/kg) produced a reduction to 28%, which was reduced to 17% following an additional 1.0 mg/kg; atropine sulphate (0.66 mg/kg) produced no further reduction.

In the four rabbits in which the effect of hemicholinium on blood pressure and respiration were measured, the initial dose (0.05 mg/kg, intravenously) produced a consistent decrease in respiratory excursions which was gradual in onset and returned to approximately the control level at the end of 1 hr. This was accompanied by a moderate decrease in blood pressure in the two rabbits in which artificial ventilation was not used, and no significant change in the other two, in which the height of the ventilatory excursions was maintained artificially. With the second dose (0.1 mg/kg in three, 0.05 mg/kg in one), given 1 hr after the first, respiratory depression was both greater and more abrupt in onset; a prolonged depressor effect occurred in all four rabbits, preceded in one by a transient pressor response which accompanied the initial period of respiratory depression. The blood pressure was only partially restored by the institution of artificial ventilation. These effects were intensified in one animal which was subsequently given injections of increased amounts of hemicholinium at various intervals to a total of 2.6 mg/kg, while under constant artificial ventilation. During this period the blood pressure fell from a control level of 110/95 to 47/35 mm Hg before sacrifice. In the two rabbits of this series in which contractions of the nictitating membrane were recorded, in one tension fell to approximately the same levels as the means after corresponding doses in the animals represented in the curves in Fig. 2, and by about one-half this extent in the other. No significant decrease was noted in the hypertensive responses to noradrenaline after 0.05 or 0.10 mg/kg of hemicholinium in the three rabbits given these amounts, or after the cumulative dose of 0.80 mg/kg in the fourth rabbit. The depressor response to acetylcholine was diminished only in those instances when the blood pressure was already at a low level.

In confirmation of Wilson & Long (1959), hemicholinium in the dosage range of 0.05 to 1.0 mg/kg, intravenously, had no significant effect on the contractions of the nictitating membrane produced by ganglionic stimulation in the three cats.

DISCUSSION

The principal finding in the present study is that hemicholinium (0.05 or 0.10 mg/kg, intravenously) produced a considerable, reversible reduction in the isometric contraction of the nictitating membrane of the rabbit in response to supramaximal, tetanic stimulation of the postganglionic sympathetic trunk. With this dosage range, the drug caused reduc-

tion in the height of respiratory excursions, inconsistent hypotension which was at least partially corrected when respiration was supported artificially, and no significant blockade of the cardiovascular effects of noradrenaline or acetylcholine. Contraction of the postganglionically stimulated rabbit nictitating membrane was also reduced by approximately 70% following phentolamine (2.0 mg/kg, intravenously), which supports the assumption that these fibres are predominantly or essentially adrenergic; the additional administration of atropine sulphate (0.5 to 0.66 mg/kg, intravenously) caused slight or no further reduction.

In contrast to that of the rabbit, the postganglionically stimulated nictitating membrane of the cat was found to be extremely resistant to hemicholinium (0.05 to 1.0 mg/kg, intravenously), as had been shown previously (Wilson & Long, 1959). Inasmuch as the primary known action of hemicholinium on autonomic neuroeffector systems is interference with the synthesis and release of acetylcholine (MacIntosh, 1959; Long, 1961), these results suggest that acetylcholine may be involved in the release of noradrenaline by the postganglionic sympathetic fibres that innervate the nictitating membrane in the rabbit, but not in the cat. The same tentative conclusion was reached from histochemical observations of the comparative amounts of acetylcholinesterase in the sympathetic ganglion cells and peripheral innervation of the nictitating membranes of the two species (Koelle, 1953, 1955; Jacobowitz & Koelle, 1965).

The great variations in the susceptibility of various cholinergic junctions to hemicholinium are well known (Long, 1961). Accordingly, while the results with hemicholinium in the rabbit appear to support the Burn & Rand hypothesis, the negative findings with this drug in the cat do not in themselves exclude the possible occurrence of a similar mechanism in the latter species, which might operate on a more restricted basis. The reported effects of cholinergic blocking and anticholinesterase agents on this organ at least suggest such a possibility. Burn *et al.* (1963a) demonstrated with the cat membrane that hyoscine (0.1 mg/kg, intravenously) produced a significant reduction in the height of contraction during supramaximal postganglionic stimulation at frequencies below 5 shocks/sec; at the latter frequency or higher, when the response was essentially maximal, little or no reduction occurred. Taken alone, these findings indicate only that the postganglionic innervation of the membrane includes both hyoscine-insensitive (that is, adrenergic) and hyoscine-sensitive (that is, cholinergic) components, both of which are excitatory. With the maximal response obtained at frequencies of 5 shocks/sec or greater, the response to the cholinergic would be masked by that to the adrenergic fibres. This was essentially the conclusion reached by Nystrom (1962) from extracellular recordings of smooth muscle action potentials in the cat nictitating membrane, where he found that single preganglionic shocks produced two negative potentials, the first of which was blocked by hyoscine, and the second by phenoxybenzamine or piperoxan, followed by a series of rhythmic fluctuations. However, in a related study, Burn *et al.* (1963b) showed that in the nictitating membrane of the hyoscine- or atropine-treated cat, where the direct action of acetylcholine on the effector cells should have been blocked, anticholinesterase agents (physostigmine or neostigmine, 0.5 mg/kg, intravenously) produced a substantial increase in the height of isotonic contraction with supramaximal postganglionic stimulation at frequencies below 5 shocks/sec. This result is most readily explained by a facilitation by acetylcholine of the release of noradrenaline. The failure of Gardiner *et al.* (1962) to obtain similar potentiation of the response with physostigmine may be attributed to the small intravenous dose given (30 μg) along with inconsistent absorption of the various amounts (5 to 500 μg) applied locally.

Nystrom (1962) reported that, of the two negative waves recorded extracellularly at the membrane, only the first (hyoscine-sensitive) wave was enhanced by physostigmine. However, in the examples shown, physostigmine was given either when the second (piperoxan-sensitive) wave was already maximal, and hence no increase could be expected, or when the stimulus strength exceeded the threshold for only the most excitable fibres, when any moderate effect could have been masked by the succeeding rhythmic fluctuations. The most consistent effect obtained with physostigmine by Bowman *et al.* (1964), who used isometric recording of membrane contraction and single shock stimulation of the post-ganglionic nerve, was a decrease in contraction height and prolongation of its time course; with repetitive stimulation, this produced an increase in tension because of an increased fusion of contractions. These investigators considered the Burn & Rand hypothesis as one among four possible interpretations of their findings.

From both the present pharmacological and the earlier histochemical comparisons on the nictitating membranes of the rabbit and cat, we suggest that, if acetylcholine participates in adrenergic transmission, as claimed by the Burn & Rand hypothesis, its role in this regard is of greater significance or extent in the rabbit than in the cat. In extension of this suggestion, the catechol amine-releasing role claimed for acetylcholine might be regarded tentatively as a facilitatory rather than as an essential step in adrenergic transmission.

SUMMARY

1. Hemicholinium (HC-3), 0.05 to 0.10 mg/kg, intravenously, produced considerable reversible inhibition of the isometric contraction of the rabbit nictitating membrane in response to supramaximal repetitive stimulation (20 shocks/sec for 10 sec) of the post-ganglionic sympathetic trunk.

2. In confirmation of previous reports, the corresponding system of the cat was highly resistant to hemicholinium (0.05 to 1.0 mg/kg, intravenously).

3. The response of the rabbit membrane was also greatly reduced by phentolamine (2.0 mg/kg, intravenously); the subsequent administration of atropine sulphate (0.50 to 0.66 mg/kg, intravenously) caused slight or no further reduction.

4. These and earlier histochemical and pharmacological findings suggest that the mediation of catechol amine release by acetylcholine in adrenergic fibres, as proposed by Burn & Rand (1959, 1962), may be of greater significance in the rabbit than in the cat, and that this hypothetical role of acetylcholine might be considered as facilitatory rather than as essential.

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