

THE EFFECT OF MUSCARINE ON PERFUSED SUPERIOR CERVICAL GANGLIA OF CATS

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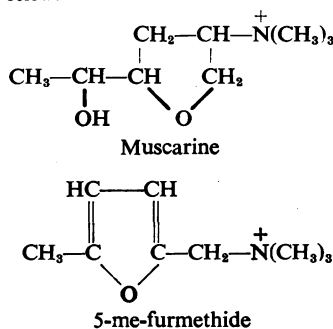
In 1914 Dale described the actions of acetylcholine as being of two distinct types, namely, muscarinic and nicotinic. He defined the muscarinic action as "the action which true muscarine exhibits in its pure form, uncomplicated by the nicotine action." The muscarine effects of choline esters were "purely peripheral in their origin, unaffected by nicotine in large doses, but readily abolished by small doses of atropine." This useful classification has been widely used ever since. With increasing knowledge of the physiology of the autonomic nervous system, it is now possible to define nicotinic effects as the effects of acetylcholine and other drugs at ganglionic synapses of both sympathetic and parasympathetic ganglia, and also at neuromuscular junctions; and muscarinic actions as actions on the effector cells innervated by postganglionic cholinergic fibres, mostly in parasympathetic nerves.

Reasons have been given previously (Ambache, 1949) for suspecting that natural muscarine may have actions other than those hitherto strictly defined as "muscarinic." During an investigation of the pharmacology of the synthetic compound 2268F (acetal of 2:3-dihydroxypropyl trimethylammonium iodide), thought to be isomeric with muscarine,* it was found that, although endowed with intense muscarinic activity, this compound also exhibited nicotinic effects on striated muscle and sympathetic ganglia. The ganglionic effect was shown to be antagonized by atropine, and the suggestion was made that in experiments on the blood pressure of atropinized cats this type of antagonism might well prevent the detection of the nicotinic effects of 2268F and of similar compounds (as in the experiments of Fournau, Bovet, Bovet, and Montezin, 1944) and possibly also of muscarine itself. An analogous situation was described later by Ing, Kordik, and Tudor Williams (1952) for furmethide (furfuryl trimethylammonium iodide) and for its 5-methyl derivative. Both these compounds have powerful muscarinic actions;

they also display some degree of nicotinic activity on the perfused superior cervical ganglion. Yet, even in large doses, neither produces a rise in blood pressure in the atropinized cat. Root's (1951) experiments with pilocarpine also suggest that a pressor action of this substance may have been masked by atropine. Furthermore, the ganglion-blocking action of atropine may antagonize even the effect of acetylcholine, as found in denervated ganglia by Konzett and Rothlin (1949). Several examples of this type of ganglionic action of atropine have been tabulated in a previous paper (Ambache, 1954). We are thus faced, firstly, with a considerable body of evidence suggesting that atropine may block not only muscarinic effects but also certain nicotinic effects of muscarine-like substances, and, secondly, with the possibility that muscarine itself may have certain nicotine-like actions.

We have therefore examined the effect of a sample of chromatographically purified, crystalline, muscarine chloride on perfused preparations of the cat's superior cervical ganglion. A preliminary account of these results has appeared elsewhere (Ambache, Perry, and Robertson, 1953). Later, Waser (1955)

* According to Eugster (1956) the empirical formula for muscarine is $C_8H_{18}O_3N$ and not, as thought by Kögl, Duisberg, and Erxleben (1931), $C_8H_{18}O_3N$. Eugster's careful work suggests that muscarine is a salt of trimethyl-2-(α -hydroxyethyl)-tetrahydro-furyl-(4)-ammonium. The resemblance to 5-methyl-furmethide is shown in the formulae below:



found that another sample of muscarine chloride prepared by Eugster and Waser (1954) had no pressor, "nicotinic," effect on the cat's blood pressure, but his experiments were conducted in the presence of atropine. When, however, Konzett and Waser (1956) examined the activity of Eugster and Waser's sample of muscarine on perfused preparations of cats' cervical ganglia, they obtained results which were identical with ours, and also showed that a subthreshold dose of muscarine may potentiate responses to acetylcholine and to preganglionic stimulation.

METHODS

Muscarine.—A sample of highly purified crystalline muscarine chloride, prepared from *Amanita muscaria* at the Wellcome Research Laboratories, was sent to us through the courtesy of Dr. S. Wilkinson and Dr. J. W. Trevan. When tested on guinea-pig ileum preparations this sample was active at a threshold concentration of 3×10^{-9} (Ambache and Lessin, 1953; 1955).

Perfusion of the Superior Cervical Ganglion.—Cats were anaesthetized with 40 mg./kg. pentobarbitone sodium intraperitoneally or, in a few experiments, with ether followed by intravenous chloralose (80 mg./kg.). The right superior cervical ganglion was prepared by the method described by Kibjakow (1933), with modifications suggested by Feldberg and Gaddum (1934), and was perfused with aerated Locke solution containing 2 g./l. of glucose and filtered through sintered glass (No. 4 Jena). The Locke solution was warmed by passage through a plastic tube

lodged in the oesophagus, as suggested by MacIntosh (personal communication). Otherwise apparatus and method were the same as used by Ambache (1949), except that the linen filter in the perfusion circuit was replaced by a small sintered glass filter. Blood pressure was recorded from a femoral artery. Intravenous injections were made into the contralateral femoral vein.

Denervated Ganglia.—In an initial aseptic operation under pentobarbitone sodium anaesthesia the right, or sometimes both, superior cervical ganglia were decentralized by avulsion of about 1 in. of the vago-sympathetic trunk low in the neck. The denervated ganglia were perfused after an interval varying from 11 days to 5 months.

RESULTS

Action of Muscarine on Normal Ganglia.—As is well known, very small doses of muscarine, given intravenously, cause a large fall in blood pressure in animals which have received no atropine. Muscarine was therefore administered to the ganglion by injection into the perfusion fluid. Injection of muscarine in this way into normal ganglia was followed by a contraction of the nictitating membrane in 14 out of 16 experiments. The effective dose of muscarine varied considerably. Thus in the fourteen experiments in which muscarine produced a contraction the doses were: in eight, 20–187 μ g.; in three, 1.6–4 μ g.; and in another three, 0.1 μ g. An experiment on one of the most sensitive preparations is illustrated in Fig. 1. In this experiment the femoral blood pres-

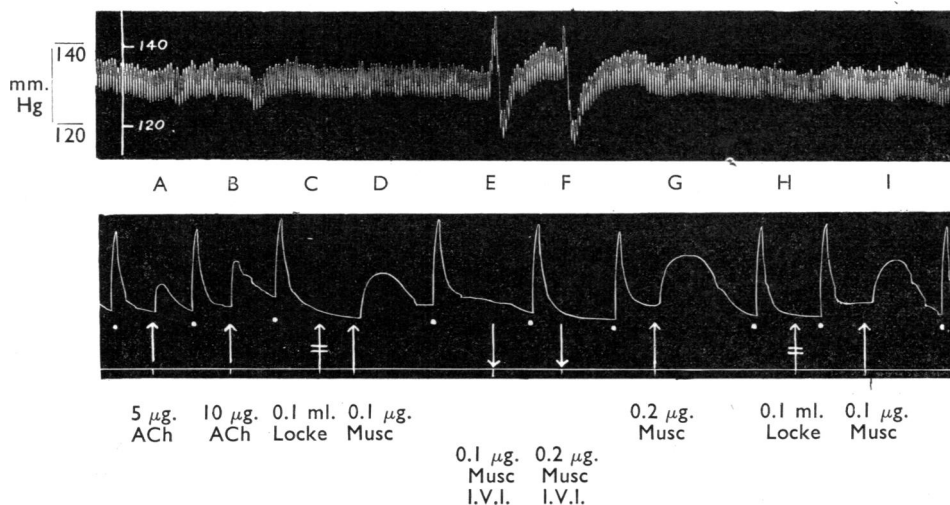


FIG. 1.—Cat, perfusion of innervated superior cervical ganglion. Effect of pure muscarine. Above, femoral blood pressure; below, contractions, upwards, of the nictitating membrane. At the dots, maximal preganglionic stimulation applied for 5 sec. Muscarine: 0.1 μ g. at D and I, and 0.2 μ g. at G, administered to the perfused ganglion; and 0.1 μ g. intravenously at E and 0.2 μ g. at F (downward arrows). Acetylcholine at A and B, and Locke solution at C and H, into the perfusion stream.

sure was also recorded. Muscarine in a dose of 0.1 μ g. produced a contraction of the nictitating membrane, which was roughly equal in size to that produced by the injection of 10 μ g. of acetylcholine, but which developed more slowly, after a longer latency (9 sec.), and which was of longer duration. In this experiment the ganglion was rather less sensitive at the start of perfusion, requiring doses of 0.5–1 μ g. of muscarine for contractions to be elicited.

This increasing sensitivity during prolonged perfusion was noticed in several other experiments. In one cat 20 μ g. of muscarine was quite without effect at an early stage of the perfusion, although later on a threshold response was obtained with 0.2 μ g. This gradual sensitization of the ganglion may be due to a cumulative effect of muscarine. In the 2 experiments in which we failed to get any response to muscarine the drug was given only at the start of the perfusion and the dose was not increased above 20 μ g. owing to the scarcity of material. From Fig. 1 it is apparent that the potency of muscarine relative to acetylcholine was of the order of 100:1, but values of this ratio varying from 200:1 to 1:20 were observed. The varying ratio could, however, almost wholly be accounted for by variations in the sensitivity to muscarine. Thus the ratio not only varied from cat to cat but also tended to increase as the perfusion proceeded in any one cat.

Control injections of Locke solution did not produce stimulation in any of the experiments.

Action of Muscarine on Denervated Ganglia.—Muscarine stimulated all of 13 ganglia which had been decentralized for periods long enough to allow degeneration of their preganglionic nerve supply. This showed that the ganglionic effect of muscarine could not be attributed to indirect stimulation of preganglionic nerve fibres and endings. As a group, the denervated ganglia appeared to be more consistent in responding to low doses of muscarine than the normal ganglia, of which only a small proportion had responded to as little as 0.1 μ g. muscarine. In fact *all* the denervated ganglia responded to small doses of muscarine—five to 0.2–0.5 μ g.; seven to 2–4 μ g.; and one to 12.5 μ g. Thus in the denervated ganglia the threshold dose for stimulation was rarely greater than 3 μ g. It appears, therefore, that the occasional relatively insensitive normal ganglion may be rendered sensitive by denervation. We did not observe

much increase in the sensitivity to acetylcholine after denervation, and, on the average, the dose required for stimulation was some one-third to one-half of that required in normal ganglia, there being a considerable overlap in the effective doses in the two groups.

Comparison with Choline.—The most common pharmacologically active impurity in extracts of *Amanita muscaria* is choline; in fact such extracts may contain 20 times as much choline as muscarine (King, 1922). Although our specimen of muscarine was believed to be pure, we tested the effects of choline injected in the same way as the muscarine (Fig. 2). It will be seen that 75 μ g. choline was required to produce a contraction equivalent in size to that produced by 0.1 μ g. muscarine. Thus, the effect of the crystalline material could not be due to traces of choline in it, since,

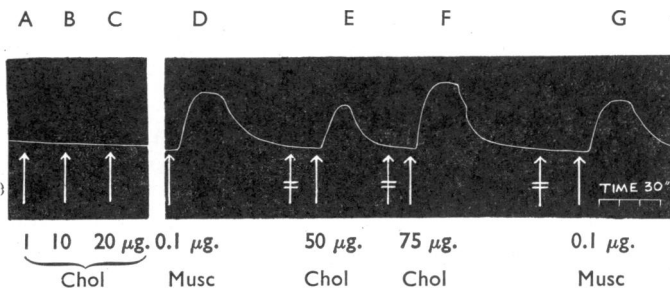


FIG. 2.—Cat superior cervical ganglion, preganglionically denervated 40 days previously. Comparison of muscarine with choline. All injections into the perfusion stream. Choline, μ g.: at A, 1; B, 10; C, 20; E, 50; and F, 75. Muscarine, 0.1 μ g. at D and G. Control injections of 0.15 ml. Locke solution (barred arrow) before E, F, and G.

even if it had consisted entirely of choline and had contained no muscarine at all, it would then have had no effect. In other experiments muscarine was 200–500 times as active as choline.

Site of Action of Muscarine.—The contractions of the nictitating membrane produced by the injection of small doses of muscarine into the perfusion stream of the isolated superior cervical ganglion were most likely to have been due to a stimulant action of muscarine on the ganglion cells. Nevertheless, the following additional evidence was obtained to show that its site of action was truly ganglionic.

It is known that the cat's nictitating membrane receives both adrenergic and cholinergic fibres from the postganglionic cervical sympathetic nerve (Bacq and Fredericq, 1935); and, indeed, the smooth muscle in the membrane responds to acetylcholine, an effect which is classifiable as muscarinic. If, therefore, muscarine were to escape out of the perfused ganglion and to reach the nictitating membrane itself, it might produce a

local contraction unrelated to any ganglion-stimulant effect. We have attempted to exclude this possibility in several ways. We have noticed in a number of experiments that it seems to be virtually impossible to achieve complete isolation of the perfused tissue from the general circulation. In about 10% of cats this is due to patency of the internal carotid artery, a condition described by Davis and Story (1943). The existence of a patent internal carotid artery can be detected by momentarily lowering the perfusion pressure to zero and observing whether there is a reflux of arterial blood into the perfusion cannula. When reflux occurred the internal carotid artery was looked for and tied. Even in the absence of a patent internal carotid, and of reflux, the ganglionic perfusate is always slightly tinged with blood when the perfusion pressure is below a certain critical level, indicating that there must be small vascular connexions between the ganglion and the general circulation. These, almost certainly, are situated in the postganglionic trunk, which has to be left intact. On raising the perfusion pressure above the critical level the venous effluent becomes clear, but the possibility then exists of some perfusate entering the general circulation. Some idea of the extent of this leak-

age into the general circulation may be gained by taking concurrent records of the blood pressure. We did, in a few experiments, observe a fall in blood pressure after injections of muscarine or of acetylcholine into the perfusion fluid; but in 5 of the experiments this was completely absent, and in general it was negligible. Thus, Fig. 1 illustrates that the administration to the ganglion of 0.2 μ g. muscarine, which elicited a large contraction of the nictitating membrane, was almost without effect on the blood pressure, whereas 0.1 μ g. muscarine, when injected intravenously, produced a considerable depressor effect and very little action on the nictitating membrane.

Further evidence for the ganglionic site of action of the muscarine in these experiments was obtained from the following study of its interaction with other drugs.

Antagonism of the Ganglionic Action of Muscarine by Atropine Administered to the Ganglia.

Atropine in very small doses blocked the stimulant action of muscarine on the ganglion. This effect was reversible and was obtained both in normal and in denervated ganglia, as illustrated in Figs. 3 and 4. In the experiment of Fig. 4, on

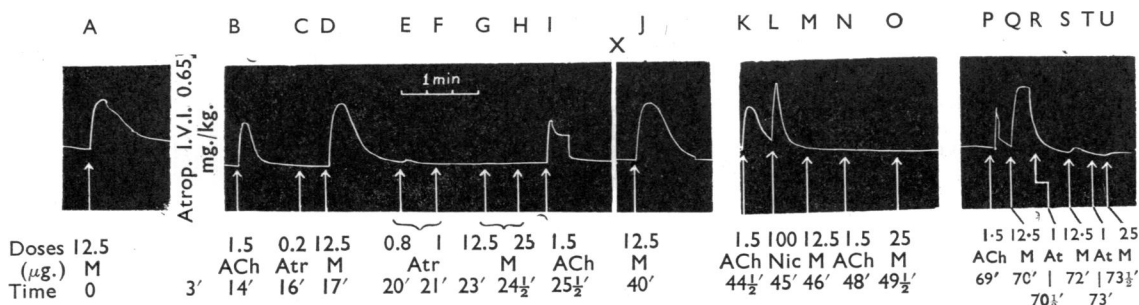


FIG. 3.—Cat, perfusion of superior cervical ganglion, 142 days denervated. Ganglionic responses to muscarine 12.5 μ g. at A, D, G, J, M, Q, and S, and 25 μ g. at H, O, and U; to acetylcholine 1.5 μ g. at B, I, K, N, and P, all injected into the perfusion system. Atropine 0.65 mg./kg. administered intravenously to the cat 11 min. before B. Atropine administered to the perfused ganglion: 0.2 μ g. at C, 0.8 μ g. at E, and 1 μ g. at F, R, and T. At L, 100 μ g. nicotine to perfusion. Drum stopped at X. Timing of doses given below in min.

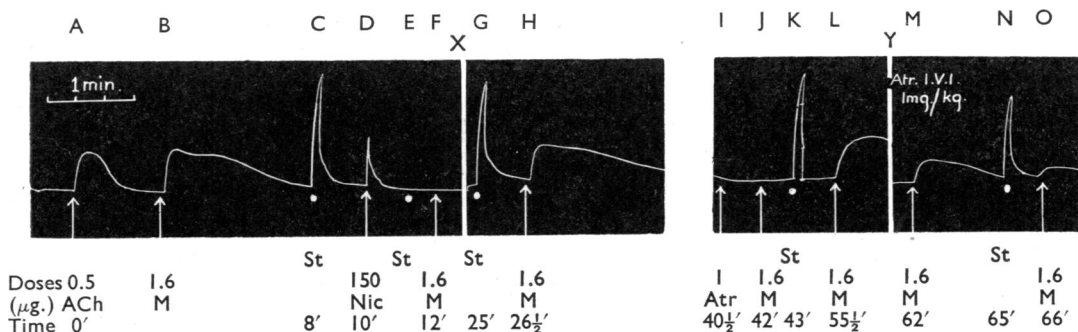


FIG. 4.—Cat, perfusion of normal superior cervical ganglion. Injections into the perfusion stream: at A, acetylcholine 0.5 μ g.; muscarine 1.6 μ g. at B, F, H, J, L, M, and O. At the white dots maximal preganglionic stimulation for 5 sec. At D, 150 μ g. nicotine, and at I, 1 μ g. atropine, administered to the ganglion. Atropine 1 mg./kg. intravenously before M (at Y). Drum stopped at X and Y; timing of doses given below in min.

a normal ganglion, after a dose of 1 μ g. atropine was administered into the perfusion fluid at I, the response to a previously effective dose of muscarine (1.6 μ g.) was completely abolished (J). This dose of atropine had no effect on the response to preganglionic stimulation (K), and its blocking effect to muscarine itself had completely passed off in 15 min. (L). A dose of 1 mg./kg. of atropine given intravenously (before M) produced, in this experiment, some depression of the response to muscarine and to preganglionic stimulation. This was attributed to some reflux, in this experiment, of atropine from the general circulation into the perfused ganglion, since in several other experiments similar doses of atropine given intravenously had no such depressant action on the ganglionic response to muscarine, as can be seen in the experiment of Fig. 3. These observations again excluded an action of muscarine on the nictitating membrane since the ganglionic effect of muscarine was obtained when the rest of the cat was atropinized, including its nictitating membranes.

Small doses of atropine did not block the effects of injected acetylcholine to the same extent as they blocked the effect of muscarine (Fig. 3); nevertheless, slightly larger doses (1–10 μ g.) did block injected acetylcholine and still larger doses (100 μ g.) could block the effects of preganglionic stimulation as well.

Suppression of Muscarine Responses by Ganglion-blocking Drugs

Normal Ganglia.—Fig. 4, D–F, shows that when transmission in the ganglion had been completely blocked by 50–100 μ g. nicotine, the stimulant effect of muscarine was also totally abolished, but recovered later when normal transmission returned. Similar effects were produced by tetramethylammonium (TMA) when this was substituted for nicotine in other experiments.

On the innervated ganglia 0.1–1 mg. hexamethonium blocked preganglionic stimulation, acetylcholine and muscarine.

Denervated Ganglia.—Preganglionic denervation produced an interesting change in the susceptibility of ganglia to certain blocking agents. Drugs which block by depolarizing ganglion cells (Paton and Perry, 1953), such as nicotine and TMA, never failed to block the ganglionic actions

of muscarine or of acetylcholine. On the other hand, hexamethonium and tetraethylammonium (TEA), which are non-depolarizing competitive blocking agents on normal ganglia, were no longer effective in blocking either acetylcholine or muscarine. Whereas in normal ganglia 0.1–1 mg. hexamethonium produced total block, in denervated ganglia doses of 10 mg. were completely ineffective. On the other hand, in denervated ganglia, relatively small doses (0.5–1 mg.) of hexamethonium and TEA still blocked the stimulant effects of nicotine, of the 3-bromophenyl ether of choline, and of TMA completely. Some of these

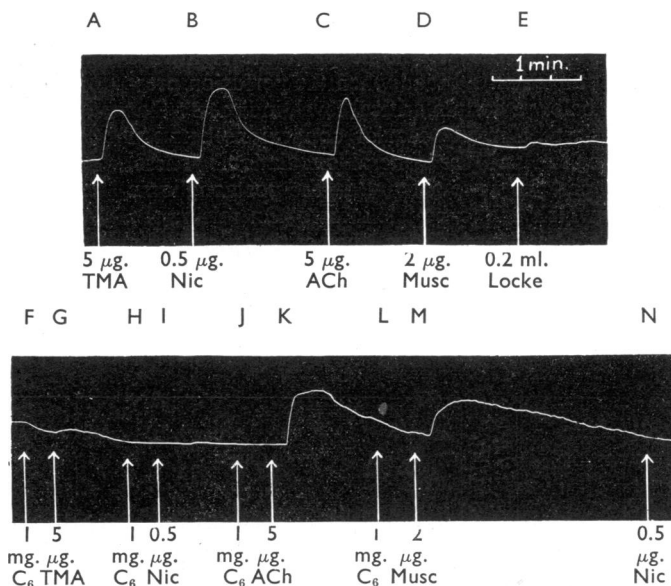


FIG. 5.—Cat, perfusion of superior cervical ganglion 15 days after preganglionic section. Stimulating effects of 5 μ g. tetramethylammonium at A, 0.5 μ g. nicotine at B, 5 μ g. ACh at C, and 2 μ g. muscarine at D. These 4 doses are repeated at G, I, K, and M, each one preceded at an interval of 1 min. by 1 mg. hexamethonium. Hexamethonium blocks nicotine and TMA, but not ACh or muscarine. Nicotine was repeated at N, 9 min. after the last dose of hexamethonium, and was still blocked. Control injection of 0.2 ml. Locke solution at E.

findings are shown in Fig. 5, which illustrates the block produced by successive doses of hexamethonium to TMA and nicotine but not to acetylcholine or muscarine. These findings were studied more extensively by Perry and Reinert (1954).

DISCUSSION

Muscarine, like acetylcholine, is capable of stimulating the ganglion cells of the cat's superior cervical ganglion. The fact that muscarine itself has such an action suggests to us that it might be preferable, in future, to use the term "parasympathomimetic" when referring to actions hitherto known as "muscarinic."

The ganglionic stimulant action of muscarine is blocked by atropine even in very small doses. Likewise, atropine has some effect, even in normal ganglia, in depressing the response to acetylcholine. The doses of atropine required are not large and the action must be regarded as a specific one. Moreover, the ganglion-stimulant effects of muscarine are also blocked, in normal ganglia, by all the usual ganglion-blocking agents, both depolarizing and competitive.

The fact that, in both normal and denervated ganglia, muscarine stimulation is abolished by nicotine is strong supporting evidence that the effect of the drug is truly ganglionic. This is also borne out by the results with other ganglion-blocking drugs. However, after denervation a remarkable change was observed, for which no simple explanation is at present forthcoming.

Hexamethonium, which before denervation completely blocked the actions of all the ganglion-stimulating drugs, failed after denervation to block acetylcholine and muscarine, although still fully effective against nicotine and TMA. This finding seems to argue that the changes in the cell membrane after denervation are such that the receptors for acetylcholine and muscarine are no longer affected by hexamethonium, while those for nicotine and TMA remain susceptible to this drug. If this is the correct interpretation, it implies that the membrane of the denervated ganglion cell is differentiated and contains at least two types of receptor. Paton and Perry (1953) have discussed the possibility that the ganglion cell membrane is differentiated into specifically reactive patches of membrane which are the site of a local depolarization, and adjacent parts of the membrane excited by electrotonic spread. It is conceivable that a similar differentiation may explain the present drug effects.

Župančič has propounded the hypothesis (see references in Župančič and Majcen, 1956) that the receptors for acetylcholine are very similar to, if not identical with, cholinesterase (ChE). According to this hypothesis, the receptor protein at sites where acetylcholine exerts a nicotinic action would resemble aceto-ChE, as both are known to be depressed by an excess of substrate. At "muscarinic" sites the receptor would resemble butyryl-ChE, as neither is inhibited by substrate excess. According to Župančič some of the changes in the pharmacology of skeletal muscle after denervation are explicable by assuming an alteration in the receptor protein towards the butyryl-ChE type, and it is conceivable that a similar change may occur in denervated ganglia. In histochemical

studies on various ganglia in cats, including the superior cervical, Koelle (1950, 1951) reported that, after preganglionic denervation, aceto-ChE had almost completely disappeared, except in occasional ganglion cells. On the other hand, the butyryl-ChE remained abundantly visible throughout the histological sections. Were the muscarinic site of action in denervated ganglion cells predominantly of the butyryl-ChE type, hexamethonium would not block muscarine or acetylcholine, since it does not block the action of these drugs at the "muscarinic receptors" in smooth muscle, which are also of the butyryl-ChE type. Moreover, Župančič has shown that atropine can block the butyryl-ChE but not the aceto-ChE; this would also fit with the results we obtained on the ganglia. Excess of nicotine, on the other hand, appears to render the ganglion cells inexcitable to all drugs, whatever their receptor sites.

Muscarine was shown by Dale and Gasser (1926) to be devoid of nicotinic action at the neuromuscular junction. We have in a few unpublished experiments confirmed this, using the frog's rectus abdominis, the leech dorsal muscle, and the pigeon's iris; Dr. E. Zaimis (personal communication) has also confirmed it in an experiment on a normal cat's tibialis anticus. Thus muscarine appears to belong to a group of drugs, all of which possess an action at ganglionic synapses but not at normal neuromuscular junctions. The other members of this group are arecoline (Dale and Gasser, 1926; Feldberg and Vartiainen, 1934), pilocarpine and acetyl- β -methyl choline (unpublished observations).

SUMMARY

1. The action of a sample of highly purified muscarine chloride, injected into perfused superior cervical ganglia of cats, has been studied.

2. Precautions were taken to reduce to a minimum the leakage of muscarine into the general circulation after injection into the ganglion perfusion. Blood pressure records showed that such leakage was absent in 5 experiments in which ganglia were stimulated by muscarine. In some of these the effective intraganglionic dose of muscarine was one which, when given intravenously, was too small to affect the nictitating membrane directly.

3. Stimulation by muscarine occurred in ganglia of which the preganglionic nerve supply had been cut and allowed to degenerate. A higher proportion of such ganglia than of normal ganglia responded to low doses of muscarine.

4. Ganglionic stimulation by muscarine persisted after atropine was administered to the cats systemically, but it was abolished reversibly by 1 μ g. or less of atropine administered to the ganglion.

5. Muscarine was 200–500 times as active as choline, thus excluding the possibility that the muscarine effect could have been due to contaminant traces of choline.

6. In normal and denervated ganglia stimulation by muscarine was always reversibly abolished by ganglionic infusion of 50–100 μ g. nicotine.

7. In normal ganglia 0.1–1 mg. hexamethonium blocked the stimulating effect of muscarine, acetylcholine, nicotine, and other drugs.

8. In denervated ganglia 1–10 mg. hexamethonium failed to block muscarine or acetylcholine, though it blocked equiactive doses of nicotine, tetramethylammonium, and the 3-bromophenyl ether of choline.

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