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The ganglion-stimulating effects of some amino-acid esters

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1. The effects of some amino-acid esters possessing both muscarinic and nicotinic activity have been investigated on the cat superior cervical ganglion. The compounds were applied to the ganglion *in vivo* by close intra-arterial injection and responses were measured by contraction of the nictitating membrane or by recording post ganglionic compound action potentials.

2. The esters, like carbachol, produced effects which were blocked by hexamethonium and reduced by atropine. Pretreatment with an anticholinesterase agent had no effect on the response and the chronically denervated ganglion was, if anything, slightly more sensitive to the drugs.

3. Effects of standard muscarinic and nicotinic drugs were also studied and the results support the view that both muscarinic and nicotinic receptors are present in the ganglion.

4. From the results of this investigation it appears that the amino-acid esters and carbachol produce their effects by interacting with post synaptic receptors in the ganglion. This is in contrast to other results in the literature which suggest that the receptors might be presynaptic. Further studies are required to resolve this difference.

The original classification by Dale (1914) of the effects of acetylcholine into nicotine-like and muscarine-like has proved to be a most useful concept but it has become increasingly apparent that pharmacological actions at the so-called nicotinic and muscarinic sites are more complex than this simple division might imply.

A revision has had to be made of the assumption that all drugs which stimulate autonomic ganglia resemble nicotine in their actions and there is strong evidence to suggest the presence of more than one type of acetylcholine receptor in the ganglia. Ahlquist & Levy (1962) divided ganglion-stimulants into two categories. The effects of Category I agents, represented by dimethylphenylpiperazinium iodide (DMPP) were blocked by classical ganglion-blocking agents such as hexamethonium, whereas the stimulatory actions of Category II drugs, such as pilocarpine or neostigmine, were blocked by atropine but somewhat potentiated by hexamethonium. This suggested the presence of muscarinic, as well as nicotinic, receptors in the ganglia, a supposition which was supported by the work of Jones (1963) which showed that muscarinic substances, including muscarine itself, produced a ganglionic response which was unaffected by hexamethonium but blocked by atropine.

Takeshige & Volle (1962, 1963) studied the action of the natural transmitter substance, acetylcholine, on the superior cervical ganglion of the cat. These authors were able to show that after pretreatment of the ganglion with anticholinisterase drugs, or after conditioning with repetitive preganglionic stimulation, acetylcholine evoked a characteristic biphasic pattern in the post-ganglionic action potentials. The " early " phase was blocked by hexamethonium and the " late " phase by atropine. Again, the authors postulated the presence of two different types of acetylcholine receptor.

In other studies Koelle & Volle (1961) claimed that carbachol, a drug with both muscarinic and nicotinic activity, stimulated the superior cervical ganglion of the cat by causing a release of acetylcholine from the presynaptic terminals. The acetylcholine so released then activated the post-synaptic membrane.

The pharmacology of a series of amino-acid esters has been described by Barrass, Brimblecombe, Parkes & Rich (1968). These substances exhibited, in the cat, a marked hypotensive effect which was blocked by atropine. On other pharmacological preparations the compounds proved to have a typical depolarizing type of action at the neuromuscular junction and also showed ganglion-stimulating activity. It seemed of interest to examine in detail the actions at the ganglia of these compounds which possess muscarinic as well as nicotinic activity.

Methods

All experiments were performed on cats weighing between 1.8 and 2.5 kg. Anaesthesia was induced with halothane and then chloralose was given by slow intravenous injection into the cephalic vein of the forelimb.

Following intubation of the trachea, a deep cervical well was prepared by forward reflection of the oesophagus and larynx. The cervical sympathetic trunk was dissected free from the vagus nerve and cut at a point about 1.5 cm. caudad to the superior cervical ganglion. The common carotid artery was dissected away from the vagus and some small branches were tied off. The lingual artery was tied and a polyethylene cannula inserted into the central end of the external carotid artery. When injections were made the common carotid artery was occluded for the period of the injection but opened immediately after. The injected material was then directed towards the superior cervical ganglion passing mainly along the internal carotid artery. Stimulation of the ganglion by a drug resulted in a contraction of the nictitating membrane which was recorded on a pen recorder (E and M Physiograph) using an E and M Myograph Type C (maximum sensitivity 5 g). The resting tension on the nictitating membrane was 1 g.

The cervical well was filled with liquid paraffin and for preganglionic stimulation the cut cervical sympathetic chain was placed on a pair of shielded silver electrodes. Supramaximal electrical stimulation was applied using an Attree stimulator. The standard stimuli were rectangular pulses of 0.5 V, 4 msec duration and 12 c/s.

In some experiments recordings of action potentials were made from the external carotid branch of the post-ganglionic nerve. This nerve was cut and placed on platinum electrodes. The action potentials were amplified using a Tektronix Type 122 AC coupled preamplifier (frequency band 80–1,000 c/s) and displayed on a cathode ray tube oscilloscope. The signals were stored on magnetic tape and subsequently photographed.

Where required, denervation of the superior cervical ganglion was carried out under pentobarbital anaesthesia (nembutal 35 mg/kg intraperitoneally). The cats were premedicated, 30 min before operation, with atropine sulphate 0.5 mg/kg intraperitoneally and promazine hydrochloride (Sparine) 1 mg/kg intramuscularly. A 1 cm segment of the cervical sympathetic trunk was resected about 3 cm caudal to the ganglion. The wound was closed and the animal treated with penicillin. Experiments were carried out, in the manner described above, 14–30 days later.

In all preparations the right femoral vein was cannulated for injection of additional anaesthetic as required. The drugs used were acetylcholine chloride, atropine sulphate, hexamethonium bromide, oxotremorine (1-[2-oxopyrrolidino]-4-pyrrolidino butyne-2), arecoline hydrochloride, carbachol (carbamylcholine chloride), dimethylphenyl-piperazinium iodide (DMPP), diisopropyl phosphorofluoridate (dyflos), tetramethylammonium iodide (TMA).

The following amino-acid esters were studied: ethyl β-dimethylaminopropionate methiodide, methyl β-dimethylaminopropionate methiodide, methyl γ-dimethylaminobutyrate methiodide, ethyl β-dimethylaminobutyrate methiodide.

All doses are expressed in terms of the salts and refer to intra-arterial injections unless otherwise stated.

Results

(a) *Effects of amino-acid esters on normal ganglia*

Doses of between 2 and 10 μg (depending on the sensitivity of the preparation) of all the esters tested caused contractions of the nictitating membrane. These contractions were completely or almost completely blocked by hexamethonium given intra-arterially (1 mg, 1 min before ester) or intravenously (2 mg, 5 min before the ester). The contractions were sometimes blocked completely and always reduced in size by pretreatment with atropine sulphate (5 μg intra-arterially 1 min before or 2.5 mg/kg intravenously 10 min before the ester). These effects are shown in Fig. 1. The esters themselves were without effect on the contraction of the nictitating membrane produced by preganglionic nerve stimulation. Hexamethonium, in the doses mentioned above, blocked these electrically-induced contractions of the nictitating membrane but atropine was without effect on the contractions.

In other experiments, illustrated in Fig. 2, post ganglionic action potentials were recorded. Typically, the duration of the asynchronous nerve discharge corresponded well with the duration of contraction of the nictitating membrane. The discharge was blocked completely by hexamethonium and reduced both in extent and duration by atropine. Similar effects were seen with methyl γ-dimethylaminobutyrate methiodide.

(b) *Effects of amino-acid esters and acetylcholine on ganglia pretreated with DFP*

An intra-arterial injection of 3 μmoles dyflos was given 15 min before administration of the amino-acid esters. Results, shown in Fig. 3, indicated that this pretreatment with dyflos made little or no difference to the sensitivity of the ganglion, the nictitating membrane contraction in response to ethyl β-dimethylaminopropionate methiodide being unaltered in strength or duration. The sensitivity of the

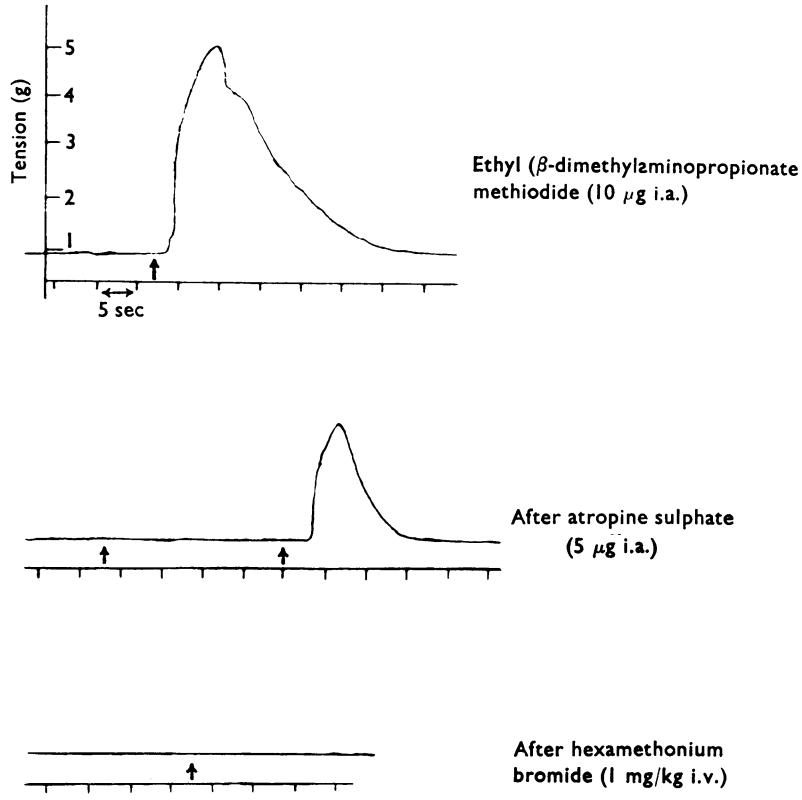

FIG. 1. Effect of ethyl β-dimethylaminopropionate methiodide on contraction of cat nictitating membrane.

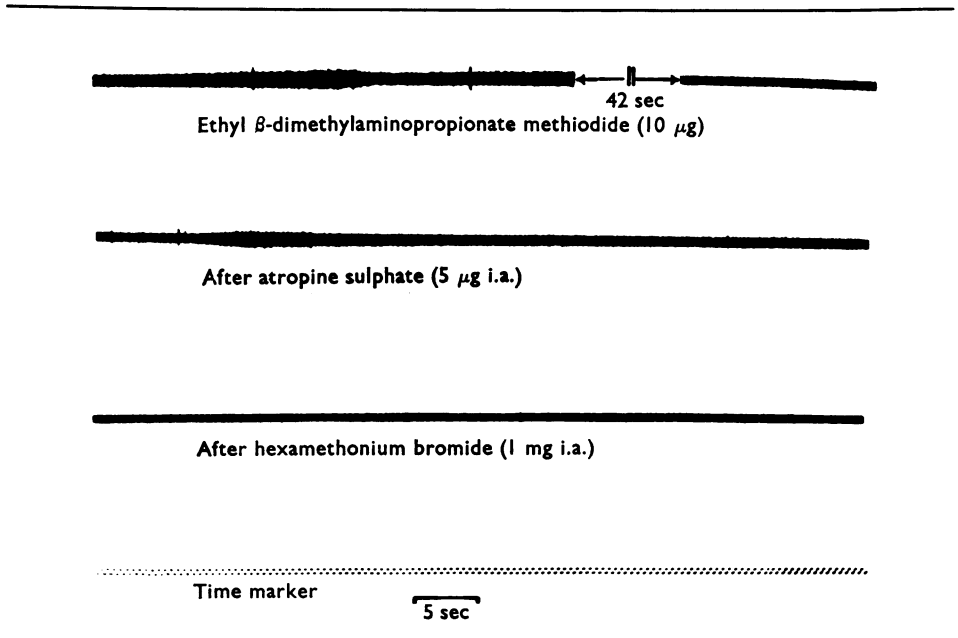

FIG. 2. Post-ganglionic discharge evoked by ethyl β-dimethylaminopropionate methiodide.

dyflos-treated ganglion to acetylcholine was, however, increased about 100 times (threshold decreased from about 10 μg to 0.1 μg).

(c) *Effects of amino-acid esters on denervated ganglia*

The contractions of the nictitating membrane produced by doses of 2–10 μg of the amino-acid esters applied to denervated ganglia were longer in duration than contractions following administration of similar doses to normal animals. This is shown in Fig. 4. The asynchronous discharge recorded from the postganglionic nerve also persisted for a longer period in denervated than in normal ganglia. The discharge was reduced in magnitude and duration by atropine and completely blocked by hexamethonium (Fig. 5).

(d) *Effects of nicotinic drugs on normal ganglia*

The two drugs chosen as typical nicotinic substances or classical ganglion stimulants were DMPP and TMA. These were used in preference to nicotine itself because there is a lesser tendency for their efforts to pass from stimulation to blockade and in this sense they appeared to show a greater similarity to the amino-acid esters than did nicotine.

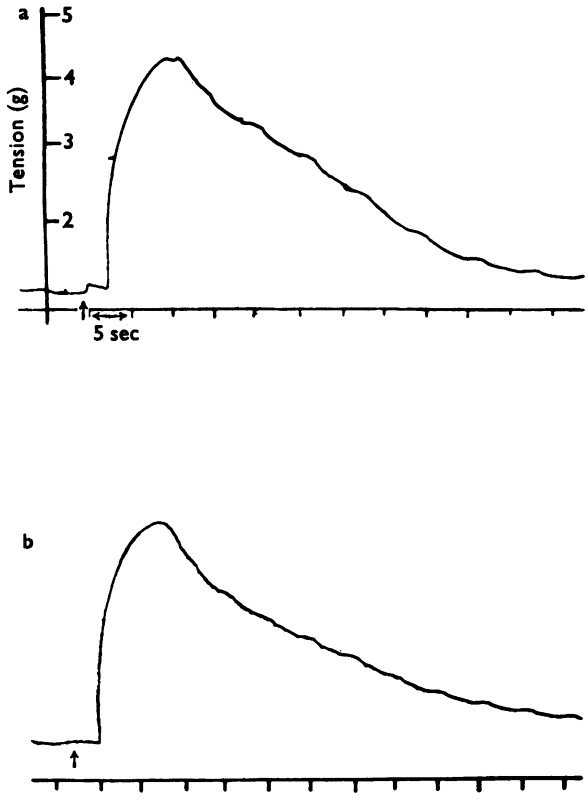

FIG. 3. Effect of pretreatment with dyflos (3 μM intra-arterially) on the response of the cat nictitating membrane to ethyl β-dimethylaminopropionate methiodide (5 μg intra-arterially), a, before dyflos; b, after dyflos.

Results obtained with DMPP are shown in Fig. 6. The contraction of the nictitating membrane produced by this drug was unaffected by atropine but completely blocked by hexamethonium. A dose of 100 μg of TMA gave a very similar result.

(e) *Effects of muscarinic drugs on normal ganglia*

Two muscarinic drugs were used, oxotremorine and arecoline. Figure 7 shows that the nictitating membrane contraction following 20 μg of oxotremorine was

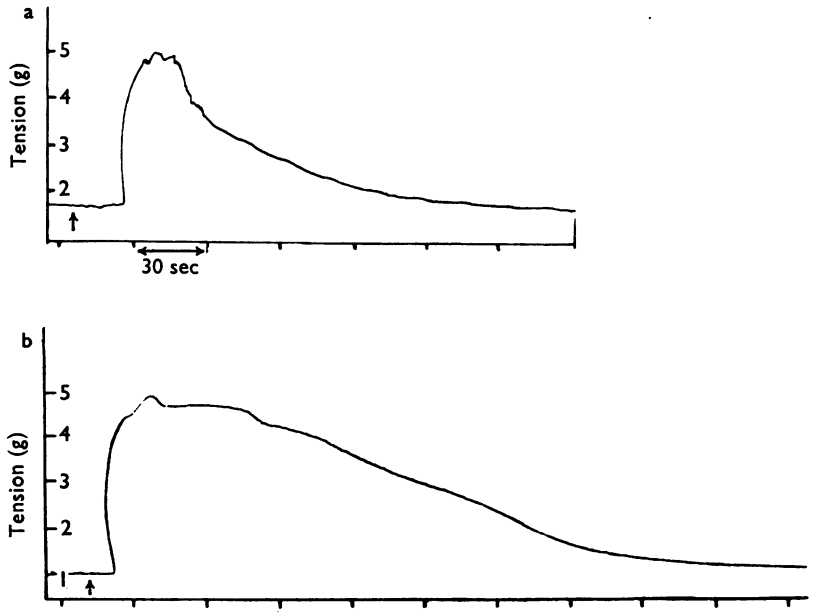

FIG. 4. Effect of methyl γ-dimethylaminobutyrate methiodide (10 μg intra-arterially) following denervation of superior cervical ganglion, a, before denervation ; b, after denervation.

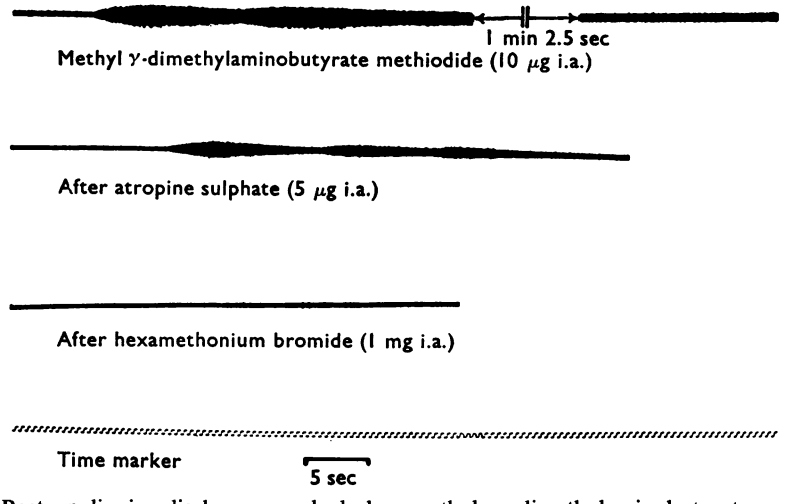

FIG. 5. Postganglionic discharge evoked by methyl γ-dimethylaminobutyrate methiodide following denervation of cat superior cervical ganglion.

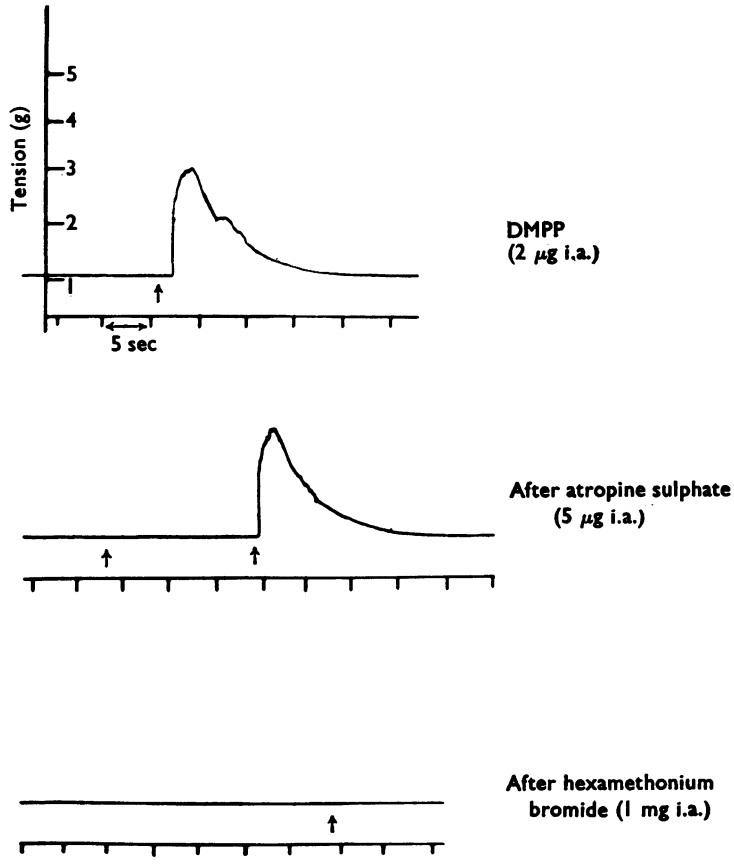

FIG. 6. Effect of DMPP on contraction of cat nictitating membrane.

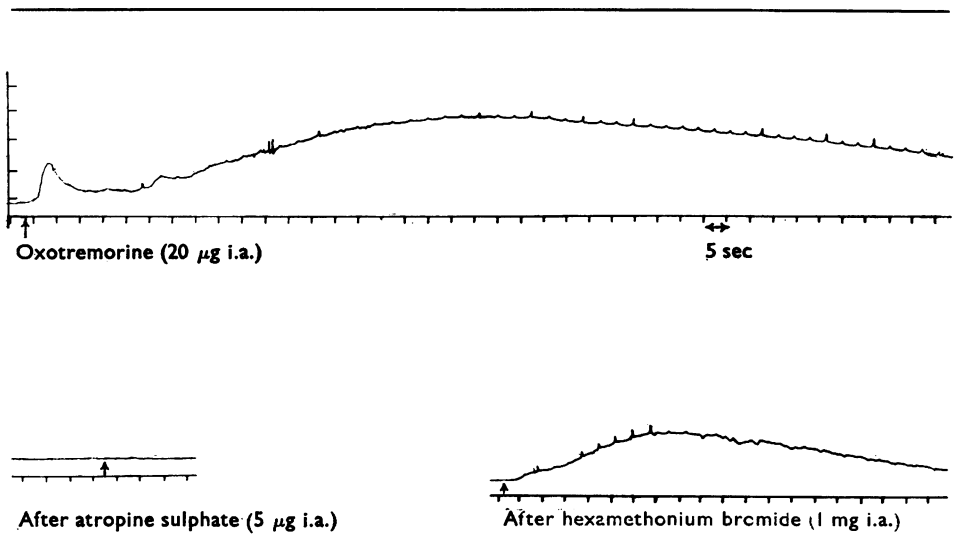

FIG. 7. Effect of oxotremorine on contraction of cat nictitating membrane.

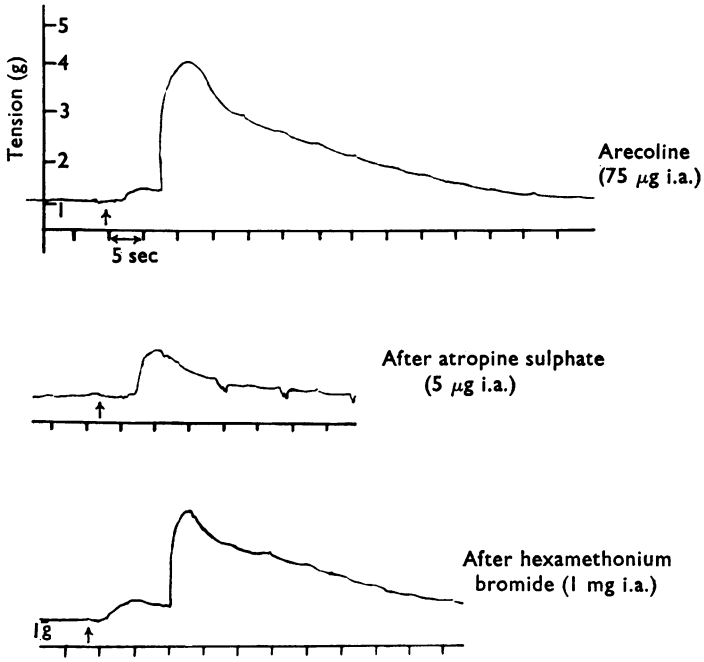

FIG. 8. Effect of arecoline on contraction of cat nictitating membrane.

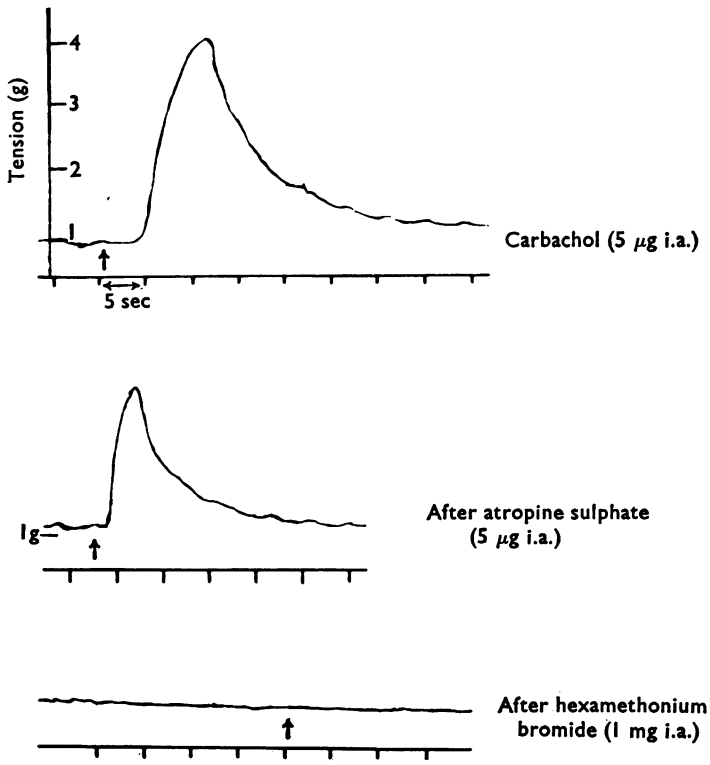

FIG. 9. Effect of carbachol on contraction of cat nictitating membrane.

completely blocked by atropine and reduced in size and duration by hexamethonium. The contraction produced by 75 μg arecoline was almost completely blocked by 5 μg atropine (a slightly larger dose of atropine blocked completely) and reduced in size and duration by hexamethonium (Fig. 8).

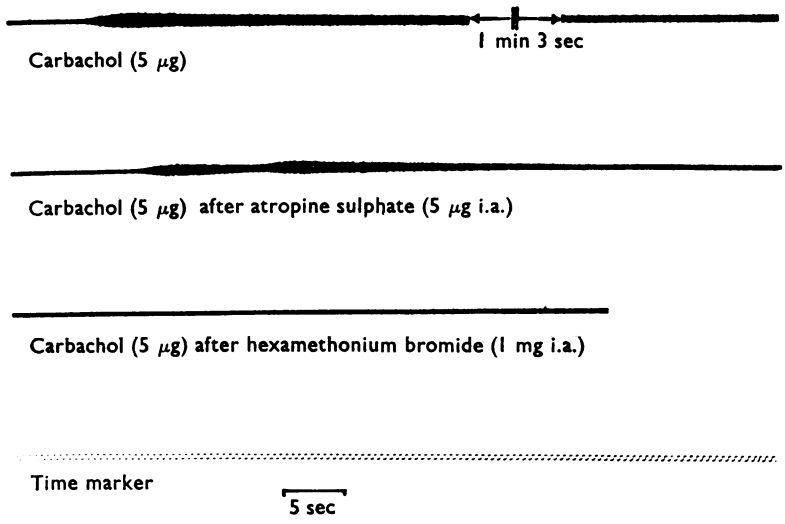

FIG. 10. Post ganglionic discharges evoked by carbachol administered intra-arterially to the superior cervical ganglion.

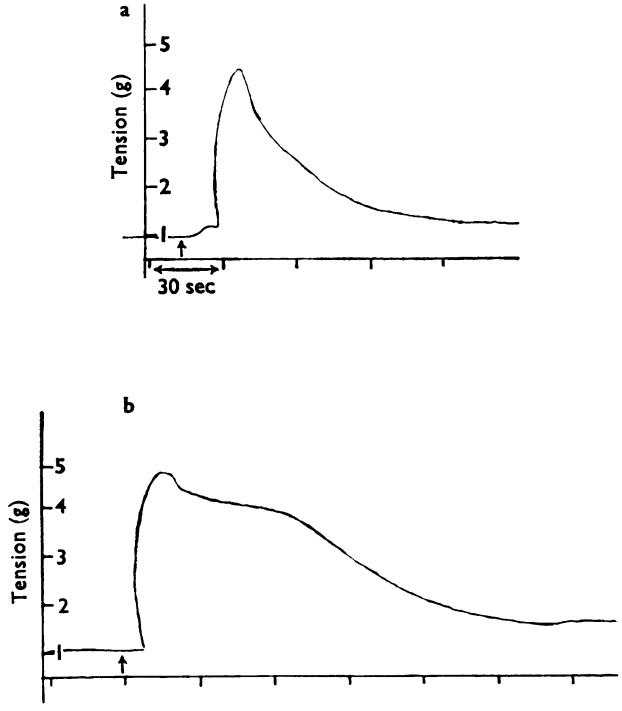

FIG. 11. Effect of carbachol (5 μg intra-arterially) on contraction of cat nictitating membrane before (a) and after (b) denervation of the superior cervical ganglion.

(f) *Effects of carbachol on normal and denervated ganglia*

Carbachol is a drug with both muscarinic and nicotinic activity. The contraction produced by 5 μg of this drug was reduced in size and duration by atropine and completely blocked by hexamethonium. These results are shown in Fig. 9 and Fig. 10 indicates that the post-ganglionic discharges showed a pattern which resembled that seen with the amino-acid esters.

After denervation of the ganglion, 5 μg of carbachol produced a response of the nictitating membrane slightly greater in magnitude and considerably longer in duration than the response produced by this dose acting on normal ganglia (Fig. 11). Again, this pattern is very similar to that seen following the amino-acid esters.

Discussion

The results presented in this report fully support the contention that there are two types of receptors present in the superior cervical ganglion. Ganglion stimulation could be produced by the administration of either muscarinic or nicotinic drugs. The effects of the latter were completely blocked by hexamethonium but were unaffected by atropine while the effects of the former were usually completely blocked by atropine but reduced in magnitude and duration by hexamethonium. It is not clear at present why hexamethonium should have any influence at all on the effects of these muscarinic drugs. This finding is not entirely consistent with that of Jones (1963) who found that hexamethonium did not block the ganglionic actions of muscarine and other muscarinic drugs. Jones, however, was using hexamethonium 3–10 mg/kg given intravenously while in our experiments 1 mg of hexamethonium was given intra-arterially and the concentration of the drug at the ganglion must have been much higher than in Jones's studies. It is of interest in this context to report that Ambache, Perry & Robertson (1956) reported a blocking action of hexamethonium against muscarine when the former was given intra-arterially. Jones (1963) suggests that this inconsistency can be explained on the bases of differences in technique and of possible weak atropine-like actions of hexamethonium.

The main object of the work reported here was to investigate the ganglionic actions of amino-acid esters with both muscarinic and nicotinic activities. Typically, these substances produced responses (contractions of the nictitating membrane or persistent asynchronous firing in the postganglionic nerve) which were completely blocked by hexamethonium and partially or completely blocked by atropine. Pretreatment of the ganglion with the anticholinesterase agent dyflos had no influence on these responses. Following chronic denervation of the ganglion the responses to the amino-acid esters were increased in magnitude and duration. An exactly similar response was produced by carbachol, a drug with both muscarinic and nicotinic activity.

The explanation of these results would seem to be that the amino-acid esters and carbachol are causing ganglion stimulation by interaction with both nicotinic and muscarinic receptors in the ganglion. The fact that dyflos potentiates the effect of acetylcholine but not that of the amino-acid esters seems to preclude any hypothesis that secondary release of acetylcholine is responsible for the observed effects of the esters. The increased sensitivity of the denervated ganglion has been reported previously by Ambache, Perry & Robertson (1956), Konzett & Waser (1956) and Jones (1963), but no simple explanation for the phenomenon is apparent.

The results given here seem to be at variance with those reported by Koelle & Volle (1961). These authors suggested that carbachol was acting at preganglionic receptors to cause the release of transmitter substance, presumably acetylcholine, which then interacted with post ganglionic receptors of the nicotinic and muscarinic type. Their evidence in favour of this hypothesis is varied but rests basically on the reported fact that the mean threshold dose of carbachol for ganglion stimulation is 26 times higher in the denervated ganglion than in the normal ganglion while there was no difference between the threshold doses of acetylcholine in normal and denervated ganglia.

It is of interest to record that Brown (1968) has reported a series of experiments in which preganglionic denervation did not reduce the sensitivity of the cat superior cervical ganglion to a series of cholinesterase-resistant drugs, including carbachol.

It is often the case that such differences in experimental findings can be explained by differences in technique but careful examination of the account of the method used by Volle & Koelle has shown that the only basic difference between their method and the one described here was the way in which injections were made to the ganglion. Volle & Koelle injected drugs through a hypodermic needle into the common carotid artery while in the experiments reported here, drugs were injected by cannula into the external carotid artery. In both cases all branches of the artery except those to the ganglion were tied and it might be expected that the ganglion received the full benefit of the injection.

Perhaps a more fundamental difference is to be found in the doses of drugs used. In our studies effects were very rarely seen with doses of less than 2 μg carbachol as the chloride (equivalent to about 1.6 μg of the base) whereas Volle & Koelle report that the mean threshold dose of carbachole for normal ganglia was 2.8 mμmoles (equivalent to about 0.4 μg). Thus some of the discrepancy might be accounted for by this higher sensitivity of Volle & Koelle's preparation but the fact remains that in our hands denervated ganglia always responded well to 5 μg of the chloride (4 μg of the base) with the threshold dose often being as low as 2 μg chloride (1.6 μg base) while Volle & Koelle report that the mean threshold dose for denervated ganglia was 71 mμmoles or about 10 μg carbachol.

Another point relevant to this discussion is that dyflos had no influence on the response to carbachol. This finding is in agreement with Volle & Koelle who explain this phenomenon by postulating that because all the acetylcholinesterase is confined to the presynaptic terminals (Koelle & Koelle, 1959) it might be retained in this area by some kind of barrier and would be effective in limiting presynaptic but not postsynaptic actions of acetylcholine. If this is a valid argument, it invalidates the suggestion made earlier in this discussion that if carbachol was acting via secondary release of acetylcholine its effects would be potentiated by a cholinesterase inhibitor.

It seems certain from the results presented here and from the work of others that both muscarinic and nicotinic receptors are present in the ganglion. The amino-acid esters and carbachol appear to interact with both types of receptor. The evidence reported would support the view that these receptors are postsynaptic but there is other evidence in the literature to suggest that they may be presynaptic.

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