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CHOLINERGIC LINK HYPOTHESIS AND SYMPATHETIC TRANSMISSION AT THE NICTITATING MEMBRANE OF THE DOG

BY

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In 1959 Burn & Rand introduced the hypothesis that acetylcholine is involved in the peripheral release of noradrenaline by postganglionic adrenergic fibres. In the light of this hypothesis effects of certain drugs on the responses of the nictitating membrane to sympathetic nerve stimulation have been studied by different investigators.

Burn & Rand (1960) demonstrated that stimulation of postganglionic fibres to the nictitating membrane of cats treated with reserpine elicited a small contraction; the contractile response was abolished by atropine and augmented by physostigmine. Gardiner, Hellman & Thompson (1962), however, could not demonstrate an increased response to postganglionic stimulation after the administration of physostigmine. Furthermore, hemicholinium (HC3), which interferes with the synthesis of acetylcholine, did not modify the response of the nictitating membrane of the cat to stimulation of the postganglionic sympathetic nerve (Wilson & Long, 1959; Gardiner & Thompson, 1961). This constituted definite evidence against the participation of acetylcholine in adrenergic transmission. Jacobowitz, Johnson, Kitchner & Koelle (1965) have recently shown that hemicholinium blocks the response of the nictitating membrane of the rabbit to stimulation of the postganglionic sympathetic nerve and have suggested that, if acetylcholine participates in adrenergic transmission, its role in this respect is of greater significance in the rabbit than in the cat. In the present investigation observations have been made on the nictitating membrane of the dog.

METHODS

Mongrel dogs of either sex weighing 6–16 kg were used. The animals were anaesthetized with urethane (1.5 g/kg) injected intravenously or subcutaneously.

A tracheal cannula was inserted and artificial ventilation started with a Palmer pump. After the laryngeal and pharyngeal blood vessels had been tied the larynx and pharynx were cut with an electric cautery between two ligatures. To enable electrodes to be placed on the postganglionic fibres of the superior cervical ganglion, the muscles underlying the ganglion on one side were cut and excised if necessary. The superior cervical ganglion was then separated from the nodose ganglion of the vagus. Shielded bipolar platinum electrodes were placed under the postganglionic fibres and fixed in position. The preganglionic sympathetic nerve and the vagus nerve of the same side were cut between ligatures and the electrodes were submerged in liquid paraffin.

The postganglionic fibres were stimulated at intervals of 4 min, with rectangular pulses of 2 msec duration and supramaximal voltage. The stimuli were applied for 15 sec and the frequency varied

between 0.5–100 pulses/sec. Contractions of the nictitating membrane of the ipsilateral side were recorded with an isotonic frontal writing lever on smoked paper. The load on the lever was 2.5 g and the contractions were magnified seven-fold.

Injections were made through a polyethylene cannula in a femoral vein. The drugs were injected in a volume of 1 to 2 ml. and were washed in with 2 ml. of 0.9% NaCl solution.

Pretreatment with reserpine

Four dogs received reserpine (0.3–0.4 mg/kg subcutaneously) 24 hr before use. These dogs required smaller doses of urethane for anaesthesia (1 g/kg).

Drugs

Bretylium tosylate, guanethidine sulphate, xylocholine bromide, α,α'-dimethylethanolamino-4, 4′ biacetophenone (hemicholinium, HC3), 3, 6-di(3-diethylaminopropoxy) pyridazine dimethiodide (Win 4981), triethylcholine iodide, atropine sulphate, physostigmine salicylate, reserpine (Serpasil, Ciba), choline chloride and methylamphetamine hydrochloride were used. Their doses refer to the salt. Adrenaline was used as base and its doses are in terms of the base.

RESULTS

Effects of stimulation of the sympathetic postganglionic nerve and of adrenaline on the nictitating membrane

Stimulation of the sympathetic postganglionic nerve at different frequencies (1/sec, 2/sec, 5/sec, 10/sec, 20/sec and 50/sec) elicited increasing contractile responses of the membrane. Responses of the membrane at the highest stimulus frequency used (100/sec) were less than those at a frequency of 50/sec.

In the dog only a few millimetres of postganglionic fibres of the superior cervical ganglion are available for stimulation. The possibility of stimulating preganglionic fibres was excluded by eliciting responses in four dogs treated with hexamethonium (5 mg/kg). The contractile responses of the membrane in these experiments were not different in any respect from the control responses. This dose of hexamethonium was sufficient to block responses of the membrane to preganglionic sympathetic nerve stimulation (two experiments).

Administered at 15 min intervals, adrenaline (5–10 μg/kg) also elicited consistent and reproducible contractions.

Effect of adrenergic neurone blocking agents on the responses of the nictitating membrane to stimulation of the postganglionic sympathetic nerve and to adrenaline

Xylocholine. Xylocholine (5 mg/kg, eleven experiments) slowly injected for 5 min produced a transient small contraction of the nictitating membrane. The contractile responses of the membrane to nerve stimulation at all the frequencies were substantially inhibited 30 min after the administration of xylocholine. The inhibition of the height of contraction ranged between 84 and 92% of the control responses (Fig. 1).

Bretylium. Bretylium (5 mg/kg, seven experiments) slowly injected for 5 min also produced a transient small contraction of the membrane. Thirty minutes after the administration of bretylium, the responses to nerve stimulation were reduced. Responses at higher frequencies (10, 20 and 50/sec) of nerve stimulation were reduced more than

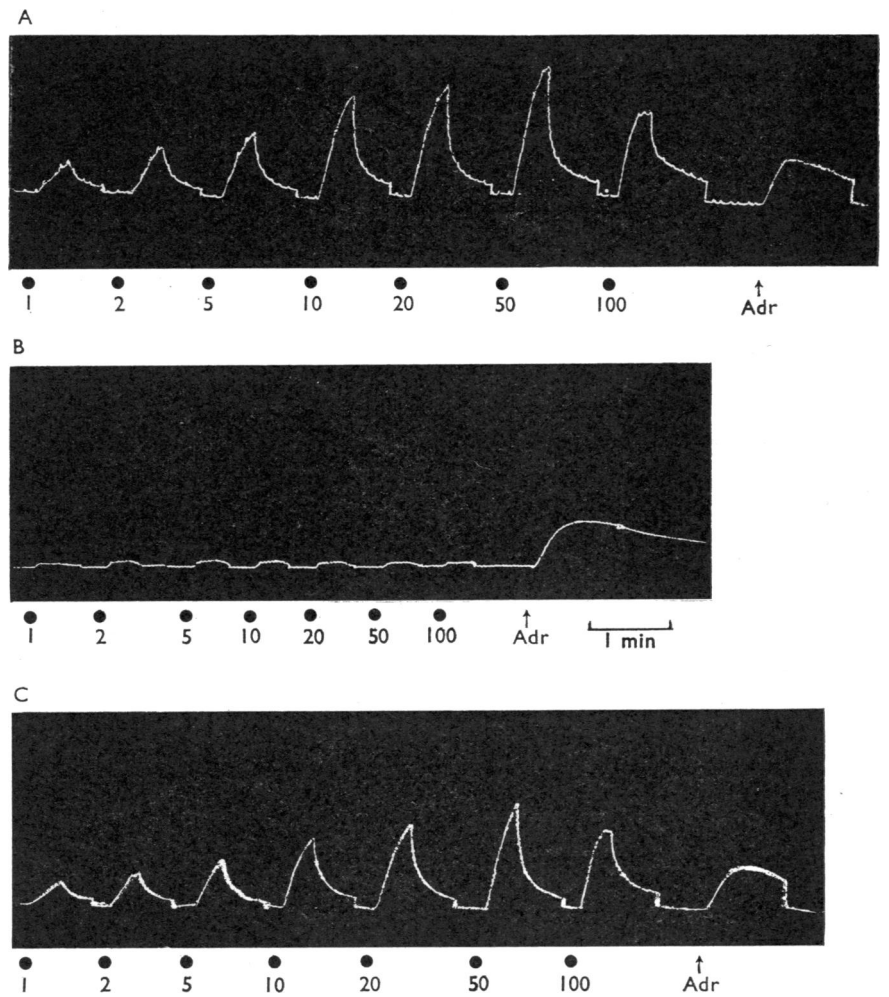

Fig. 1. Dog, 14 kg. Urethane (1.5 mg/kg) anaesthesia. Record of contractions of the nictitating membrane. Responses to stimulation of the postganglionic cervical sympathetic nerve with 1, 2, 5, 10, 20, 50 and 100 shocks/sec for 15 sec, every 4 min (dots) and to adrenaline (Adr, 10 μg/kg; arrow). Panel A shows control responses and Panel B responses 30 min after the administration of xylocholine (5 mg/kg). Methylamphetamine (0.5 mg/kg) was given 90 min after the administration of xylocholine and 150 min before recording the responses shown in Panel C. Time mark, 1 min. Injections were intravenous.

those at lower frequencies (1, 2 and 5/sec, Fig. 2). The magnitude of block at lower frequencies of nerve stimulation (1, 2 and 5/sec) ranged between 68 and 72% of the control responses and at higher frequencies between 74 and 85% of control responses.

Guanethidine. Guanethidine (2.5 mg/kg, eight experiments) slowly injected for 5 min also produced a slight contraction which was quickly followed by recovery. Responses to nerve stimulation were reduced 30 min after the administration of guanethidine. Responses to nerve stimulation at lower frequencies (1, 2 and 5/sec) were reduced more

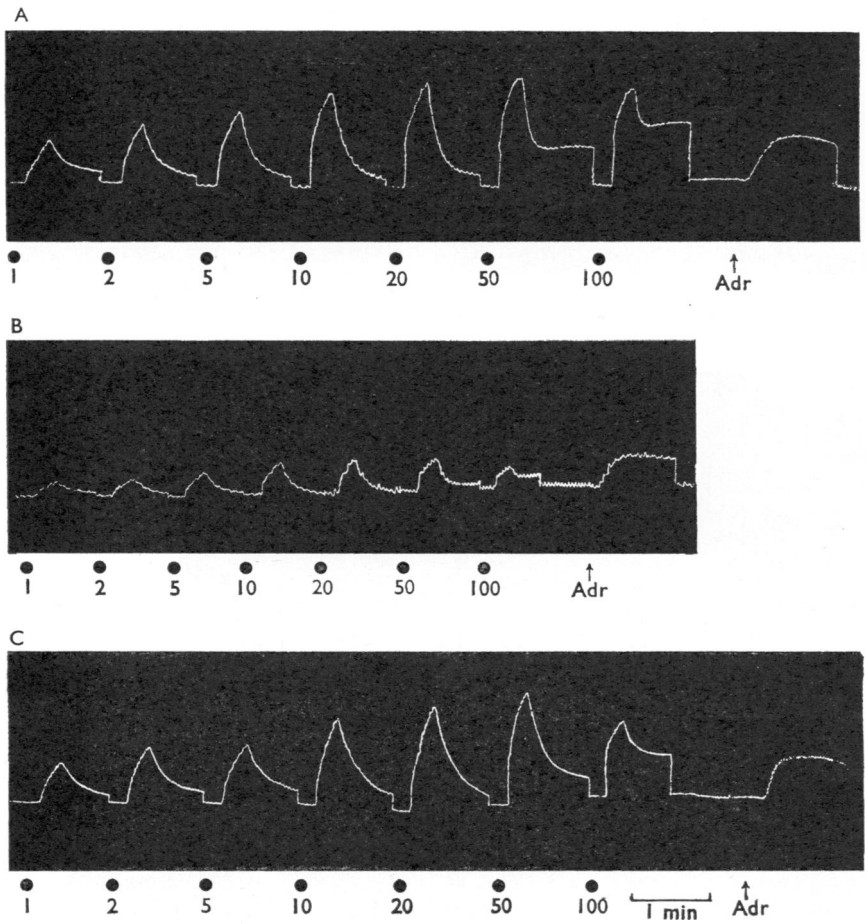

Fig. 2. Dog, 10 kg. Urethane (1.5 g/kg) anaesthesia. Record of contractions of the nictitating membrane. Responses to stimulation of the postganglionic cervical sympathetic nerve with 1, 2, 5, 10, 20, 50 and 100 shocks/sec for 15 sec, every 4 min (dots) and to adrenaline (Adr: 10 μg/kg; arrow). Panel A shows control responses and Panel B shows responses 30 min after the administration of bretylium (5 mg/kg). Methylamphetamine (0.4 mg/kg) was given 90 min after the administration of bretylium and 150 min before recording the responses shown in Panel C. Time mark, 1 min. Injections were intravenous.

than those at higher frequencies (10, 20 and 50/sec, Fig. 3). The magnitude of block at lower frequencies of nerve stimulation (1, 2 and 5/sec) ranged between 72 and 89% of the control responses and at higher frequencies between 53 and 62% of the control responses.

In two control experiments with each drug, bretylium, xylocholine and guanethidine, responses to nerve stimulation remained blocked over a period of 6 hr and longer. When the responses to nerve stimulation were markedly inhibited by xylocholine and guanethidine, responses to adrenaline (10 μg/kg) were either unaffected or were larger than the control responses. After bretylium, there was a slight inhibition of responses to adrenaline (10 μg/kg).

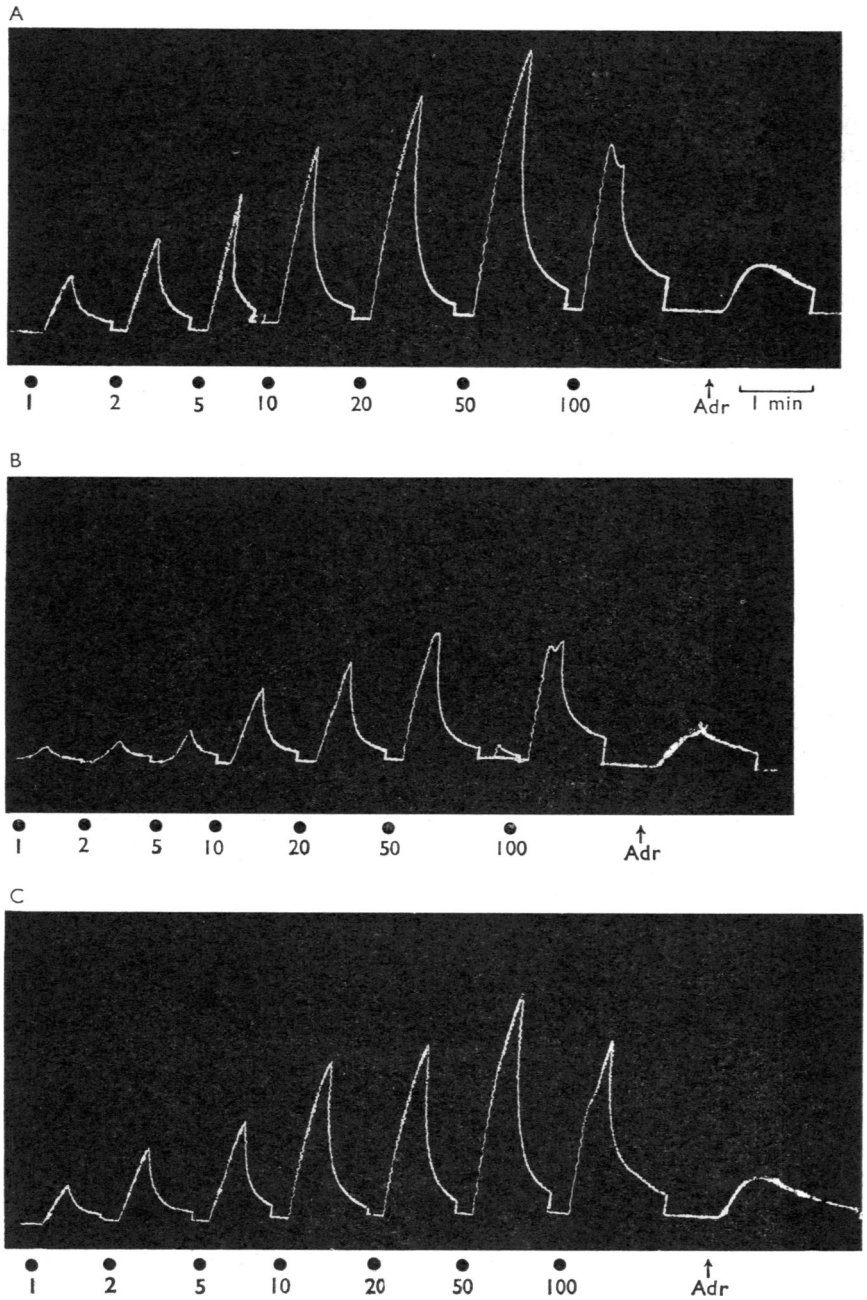

Fig. 3. Dog, 12.5 kg. Urethane (1.5 g/kg) anaesthesia. Record of contractions of the nictitating membrane. Responses to stimulation of the postganglionic cervical sympathetic nerve with 1, 2, 5, 10, 20, 50 and 100 shocks/sec for 15 sec, every 4 min (dots) and to adrenaline (Adr: 10 μg/kg; arrow). Panel A shows control responses and Panel B shows responses 30 min after the administration of guanethidine (2.5 mg/kg). Methylamphetamine (0.4 mg/kg) was given 90 min after the administration of guanethidine and 180 min before recording the responses shown in Panel C. Time mark, 1 min. Injections were intravenous.

Reversal of the blocking action of adrenergic neurone blocking agents by methylamphetamine

Methylamphetamine (0.4 mg/kg) caused a marked contraction of the membrane for more than 60 min and reduced its responses to nerve stimulation by about 25%. Responses to adrenaline were unaffected (two experiments).

Sixty to ninety minutes after the development of block of responses to nerve stimulation by xylocholine, bretylium and guanethidine, methylamphetamine (0.3–0.5 mg/kg) was given. Responses of the membrane to nerve stimulation were generally restored 150–180 min after the administration of methylamphetamine. The block was completely restored in two experiments with each of the following drugs: xylocholine, bretylium and guanethidine. The restoration was partial (35%–80%) in five of seven experiments with xylocholine, three of five experiments with bretylium and four of six experiments with guanethidine. In two experiments with xylocholine no restoration was demonstrable. Finally, in two experiments xylocholine (5 mg/kg) was given 120 min after the administration of methylamphetamine (0.4 mg/kg); responses of the membrane to nerve stimulation elicited 30 min after the administration of xylocholine were not reduced, nor were the responses to adrenaline. The results of representative experiments are illustrated in Figs. 1, 2 and 3.

Effects of hemicholinium, triethylcholine and Win 4981, drugs which interfere with the synthesis of acetylcholine

Thirty minutes after the administration of hemicholinium (5–8 mg/kg, five experiments), Win 4981 (5–8 mg/kg; five experiments) and triethylcholine (7–10 mg/kg, three experiments), a slow and progressive block of responses of the membrane to nerve stimulation was noted. The block was well marked after 60 to 90 min. The degree of block increased as the frequency of stimulation rose—for example, it ranged between 7 and 47% of the control response at lower frequencies (1, 2 and 5/sec) and between 25 and 85% at higher frequencies (10, 20, 50 and 100/sec).

With hemicholinium, the response to adrenaline (10 μg/kg) was somewhat reduced (10–20%) compared with the control response. Responses to adrenaline (10 μg/kg) were unaffected by Win 4981 and triethylcholine.

The nerve block with hemicholinium, Win 4981 and triethylcholine was almost completely reversed 5–15 min after the administration of choline chloride (10 mg/kg). Choline chloride itself caused a transient contraction of the membrane. Figure 4 illustrates the results of a typical experiment obtained with hemicholinium.

Effects of atropine

Thirty minutes after the administration of atropine (1 mg/kg, four experiments) the responses of the membrane were reduced only at lower frequencies of nerve stimulation (0.5, 1, 2 and 5/sec) but not at higher frequencies (10, 20, 50 and 100/sec). The reduced responses were restored 30 min after the administration of physostigmine (0.05 mg/kg, Fig. 5).

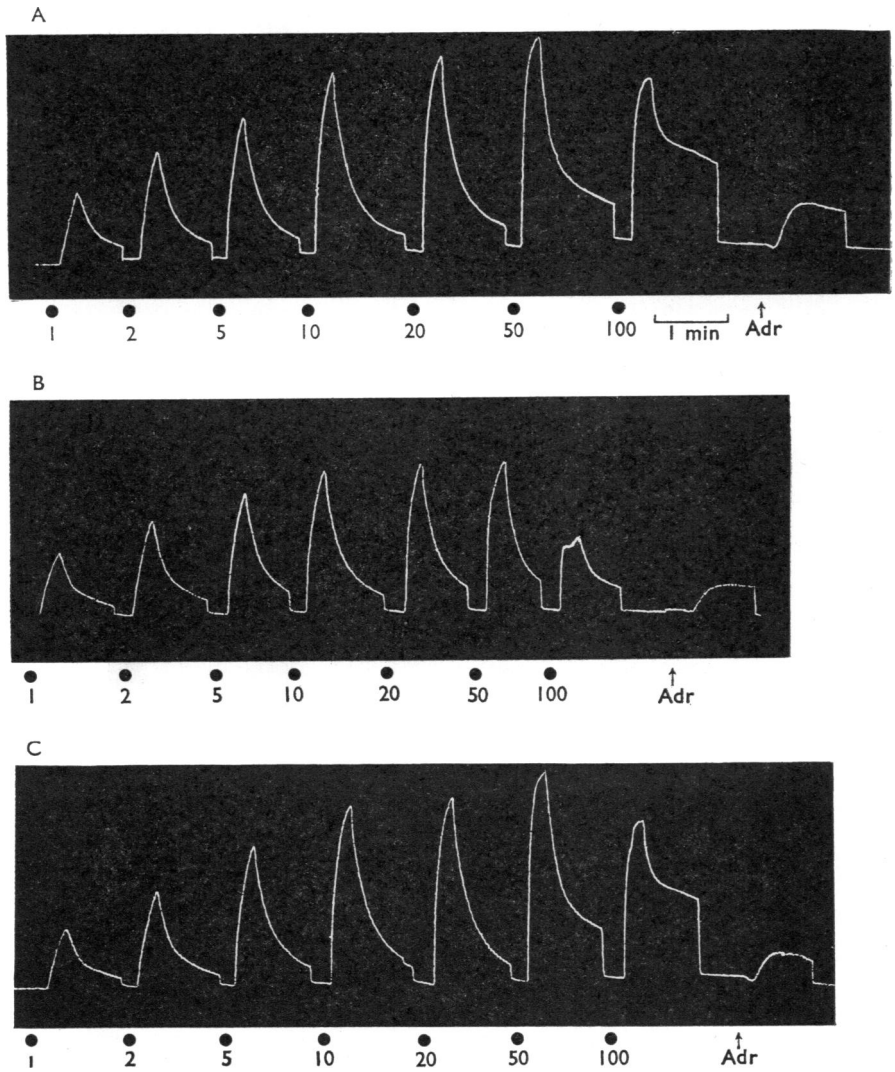

Fig. 4. Dog, 14.5 kg. Urethane (1.5 g/kg) anaesthesia. Record of contractions of the nictitating membrane. Responses to stimulation of the postganglionic cervical sympathetic nerve with 1, 2, 5, 10, 20, 50 and 100 shocks/sec for 15 sec, every 4 min (dots) and to adrenaline (Adr: 10 μg/kg ; arrow). Panel A shows control responses and Panel B shows responses 30 min after the administration of hemicholinium (8 mg/kg). Choline chloride (10 mg/kg) was given 10 min before recording the responses shown in Panel C. Time mark, 1 min. Injections were intravenous.

Dogs pretreated with reserpine

Effects of atropine and physostigmine

Dogs were used 24 hr after the administration of reserpine (0.3–0.4 mg/kg). Initially higher doses (0.5 mg/kg and more) were given, but the animals developed diarrhoea, lost weight and died early in the experiment. After recording control responses of the

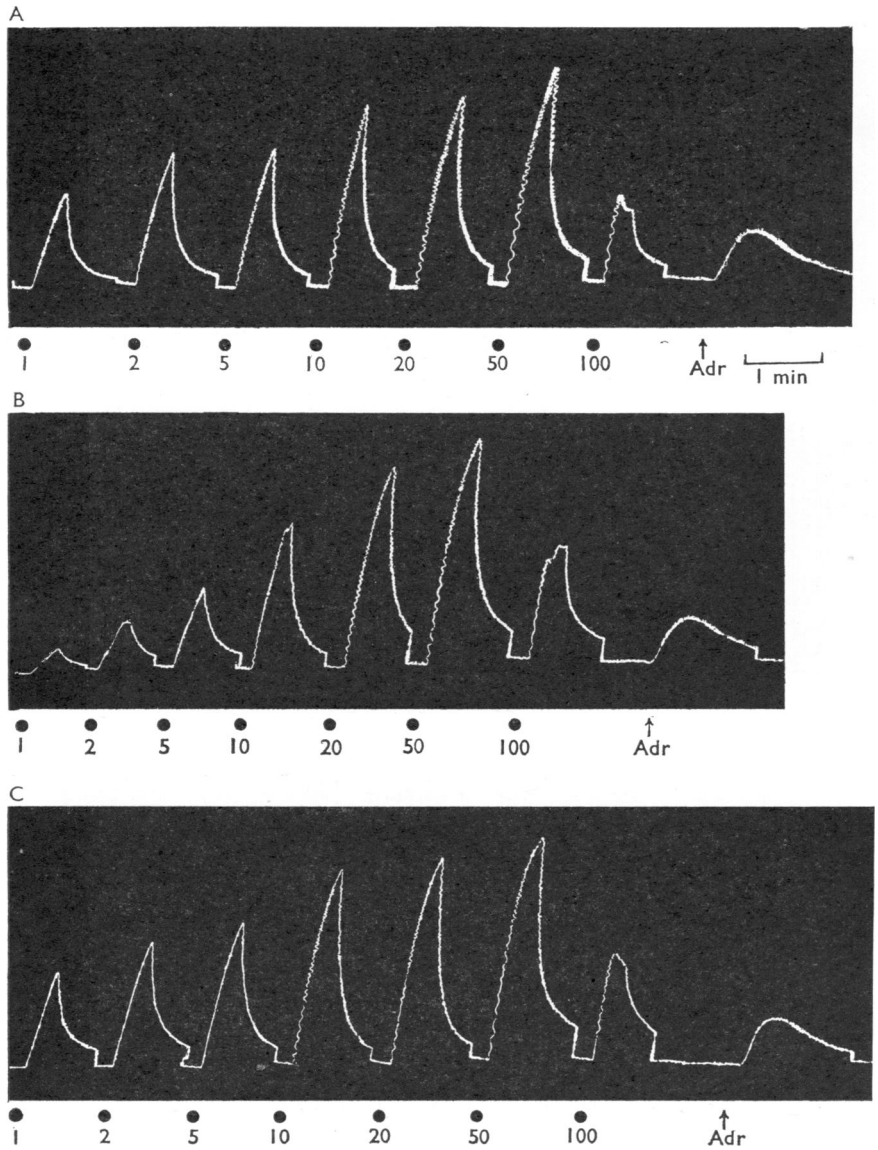

Fig. 5. Dog, 11.5 kg. Urethane (1.5 g/kg) anaesthesia. Record of contractions of the nictitating membrane. Responses to stimulation of the postganglionic cervical sympathetic nerve with 1, 2, 5, 10, 20, 50 and 100 shocks/sec for 15 sec, every 4 min (dots) and to adrenaline (Adr: 8 μg/kg; arrow). Panel A shows control responses and Panel B shows responses 30 min after the administration of atropine (1 mg/kg). Physostigmine (0.05 mg/kg) was given 30 min before recording responses shown in Panel C. Time mark, 1 min. Injections were intravenous.

membrane at different frequencies of stimulation, atropine (1 mg/kg, four experiments) was given. Thirty minutes later responses of the membrane to nerve stimulation at lower frequencies (1, 2 and 5/sec) were abolished and responses at higher frequencies (10, 20 and 50/sec) were reduced.

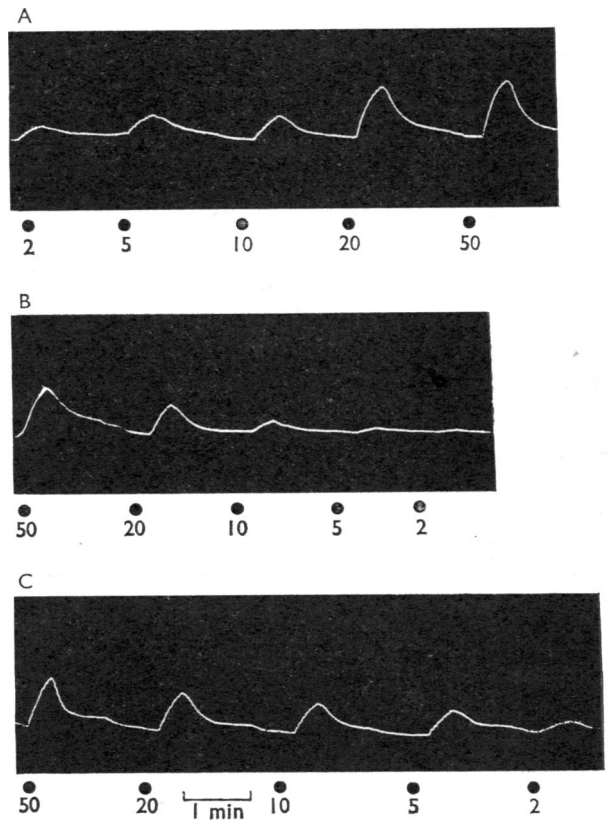

Fig. 6. Dog, 14.5 kg pretreated with reserpine (0.3 mg/kg) for one day. Urethane (1.0 g/kg) anaesthesia. Record of contractions of the nictitating membrane. Responses to stimulation of the postganglionic cervical sympathetic nerve with 2, 5, 10, 20 and 50 shocks/sec for 15 sec, every 4 min (dots). Panel A shows control responses and Panel B shows responses 30 min after the administration of atropine (1.0 mg/kg). Physostigmine 0.5 mg/kg) was given 60 min after the administration of atropine and responses shown in Panel C were recorded. Time mark, 1 min. Injections were intravenous.

Physostigmine (0.5 mg/kg) was given 60 min after the administration of atropine. There was a slow contraction of the membrane for 15 min. Responses of the membrane to nerve stimulation were fully restored (Fig. 6). The restored responses could again be blocked by atropine (1 mg/kg).

DISCUSSION

Xylocholine, bretylium and guanethidine blocked the responses of the nictitating membrane to stimulation of the postganglionic cervical sympathetic nerve. Guanethidine was more effective at lower frequencies of nerve stimulation while bretylium was more effective at higher frequencies; xylocholine was equally effective at all the frequencies. Our results on the modification of frequency responses by bretylium, guanethidine and xylocholine are in agreement with those of Boura & Green (1959), Day & Rand (1963) and Nasmyth & Andrews (1959), respectively. When the block to nerve stimulation was maximal, responses to adrenaline were either potentiated or unaffected (xylocholine and guanethidine) or were reduced (bretylium).

Acetylcholine blocks stimulation of the sympathetic postganglionic fibres to pilomotor muscles (Brücke, 1935) and the constrictor effect of sympathetic stimulation in the rabbit ear (Burn & Rand, 1960). In the presence of atropine or hyoscine, xylocholine, bretylium

and guanethidine block the sympathetic action of acetylcholine in isolated rabbit atria, and isolated rat ileum (Huković, 1960 ; Gokhale, Gulati & Panchal, 1967). Xylocholine and bretylium resemble acetylcholine chemically. Guanethidine, apparently different in structure from acetylcholine, has a guanidine group which is strongly basic and highly ionized like the trimethylammonium group of acetylcholine. If acetylcholine is assumed to be involved in the release of adrenergic transmitter from sympathetic nerve endings, the action of xylocholine, bretylium and guanethidine could be explained in terms of blocking acetylcholine.

Recently certain indirectly acting sympathomimetic amines such as dexamphetamine and certain monoamine oxidase inhibitors such as phenelzine have been shown to reverse the adrenergic nerve blocking action of xylocholine, bretylium and guanethidine (Day & Rand, 1962 ; Gokhale, Gulati & Joshi, 1965). Clinical observations also indicate that amphetamine and related drugs specifically antagonize the hypotensive action of bretylium and guanethidine (Gulati, Dave, Gokhale & Shah, 1966). In the present experiments methylamphetamine could reverse the block by xylocholine, bretylium or guanethidine of the responses to stimulation of the postganglionic sympathetic nerve.

Hemicholinium, Win 4981 and triethylcholine, which impair the synthesis of acetylcholine by interfering with the transport of choline, have been shown to produce a frequency-dependent block at the neuromuscular junction and at sympathetic ganglia (MacIntosh, Birks & Sastry, 1956 ; Wilson & Long, 1959 ; Schueler, 1960 ; Brown & Rand, 1961 ; Gesler & Hoppe, 1961), sites where the role of acetylcholine as a mediator is well established. In our experiments hemicholinium, triethylcholine and Win 4981 blocked the response to stimulation of the postganglionic sympathetic nerve. The block was slow to develop and frequency dependent in that it increased with the increase in the frequency of stimulation. The administration of choline chloride restored the responses of the nictitating membrane after block by hemicholinium, triethylcholine and Win 4981. In this respect our observations are in agreement with those of Jacobowitz *et al.* (1965) on the nictitating membrane of the rabbit but are at variance with those of Wilson & Long (1959) and Gardiner & Thompson (1961) on the nictitating membrane of the cat. The frequency dependence of the block may be the result of faster utilization of acetylcholine at higher frequencies. Thus, if acetylcholine is the penultimate mediator in adrenergic transmission, its role in this respect is of greater significance in the rabbit and dog than in the cat.

The slight reduction of the response to adrenaline after hemicholinium observed in the present experiments may be caused by some direct depressant action of hemicholinium, as has been reported for the guinea-pig vas deferens by Bentley & Sabine (1963).

Atropine reduced the contraction of the nictitating membrane at low frequencies of stimulation ; this confirms the results of Burn, Dromey & Large (1963) on the nictitating membrane of the cat. They suggest that, at low frequencies, sympathetic cholinergic fibres release acetylcholine only and that at high frequencies almost all the acetylcholine is used to mediate the release of noradrenaline.

In animals pretreated with reserpine, the responses of the nictitating membrane to stimulation of the postganglionic sympathetic nerve were greatly reduced. In such animals atropine abolished the residual response of the membrane at low frequencies of

stimulation and reduced it at high frequencies. Physostigmine reversed the block caused by atropine and sometimes the responses of the membrane to nerve stimulation at low frequencies exceeded the control responses. When atropine was given after physostigmine, the responses were again reduced ; this observation excludes the possibility that the effect of physostigmine in restoring the responses is due to the release of catecholamines from the adrenal medulla. According to Burn, Rand & Wien (1963), at low frequencies, there is time for cholinesterase to hydrolyse acetylcholine between each pulse, therefore the concentration of acetylcholine cannot rise ; however, at high frequencies there is much less time for this destruction of acetylcholine. Thus a rise in frequency has the same effect as inhibition of cholinesterase ; physostigmine, by preventing the hydrolysis of acetylcholine, exerts its greatest effect at low frequencies.

If acetylcholine participates in adrenergic transmission as suggested by Burn & Rand (1959), it should be possible to demonstrate the presence of acetylcholinesterase in the smooth muscle of the membrane. To date there is no direct evidence for the presence of cholinesterase in the postganglionic sympathetic supply to the nictitating membrane of the dog, but its presence is indicated by the observation that physostigmine reverses the block induced by atropine. Its presence in large amounts is also indicated by the rather high optimal frequency of stimulation. Burn, Rand and Wien (1963) suggested that the optimal stimulus frequency depends upon the amount of cholinesterase present. At a higher stimulus frequency cholinesterase would have less time to act and the frequency necessary to liberate a large amount of noradrenaline would depend on the amount of cholinesterase present. If there was much cholinesterase the optimal frequency would be high ; if there was very little cholinesterase the optimal frequency might be quite low. In the present experiments the optimal frequency ranged between 20 and 50 shocks/sec.

In conclusion, our observations on the nictitating membrane of the dog are consistent with the hypothesis of Burn & Rand (1959) regarding the role of acetylcholine in the peripheral release of noradrenaline by postganglionic adrenergic fibres. From the present data and the literature it seems that the role of acetylcholine in this regard is of greater significance in the dog and rabbit than in the cat.

SUMMARY

1. This study was undertaken to examine the role of a cholinergic link in sympathetic transmission at the nictitating membrane of the dog. The experiments were performed in dogs anaesthetized with urethane.

2. Xylocholine, bretylium and guanethidine blocked the responses of the nictitating membrane to stimulation of the postganglionic cervical sympathetic nerve. Xylocholine blocked the responses equally well at all frequencies of nerve stimulation (1–100 shocks/sec). Bretylium was more effective at high (10, 20 and 50/sec) than at low frequencies (1, 2 and 5/sec) while guanethidine blocked more effectively at low than at high frequencies. Responses to adrenaline were either potentiated or remained unaffected by xylocholine and guanethidine, but were slightly reduced by bretylium.

3. The block of responses of the nictitating membrane to nerve stimulation produced by xylocholine, bretylium and guanethidine was reversed either partially or completely by methylamphetamine in most of the experiments.

4. Hemicholinium, triethylcholine and 3,6-di(3-diethylaminopropoxy)pyridazine dimethiodide (Win 4981) produced a slow and progressive block of the responses of the nictitating membrane to nerve stimulation. The block was obvious after 60 to 90 min and increased with increasing frequency of stimulation. The block was almost completely reversed 5 to 15 min after the administration of choline chloride. Responses to adrenaline were unaffected by triethylcholine and Win 4981, but were reduced somewhat by hemicholinium.

5. Responses of the membrane to nerve stimulation at low (0.5, 1, 2 and 5/sec) but not at high (10, 20, 50 and 100/sec) frequencies were reduced by atropine. The reduced responses were restored by physostigmine.

6. In dogs pretreated with reserpine, atropine abolished the residual responses of the membrane to nerve stimulation at low frequencies (1, 2 and 5/sec) and reduced them at high frequencies (10, 20 and 50/sec). Physostigmine restored the responses; after physostigmine atropine again reduced the responses.

7. It is concluded that the role of acetylcholine in sympathetic transmission at the nictitating membrane is of greater significance in the dog than in the cat.

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