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USE OF THE FROG NEUROMUSCULAR JUNCTION FOR ASSESSING THE ACTION OF DRUGS AFFECTING SYNAPTIC TRANSMISSION

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In order to gain information about the possible mechanisms of action of drugs known to affect the central nervous system and transmission at ganglionic synapses, the effects of paraldehyde, methylpentynol, mephenesin and hexamethonium on neuromuscular transmission have been compared with those of tubocurarine and dimethyltubocurarine. The present work forms an extension of that of Nicholls & Quilliam (1956).

METHODS

The M. extensor longus digiti IV of the frog was used in the manner described by Fatt (1950) and Nicholls & Quilliam (1956). The effects of the drugs on nerve action potentials, miniature end-plate potentials and muscle action potentials elicited by direct stimulation were estimated as described by Payton & Shand (1961). Paraldehyde (B.D.H.), methylpentynol (British Schering), mephenesin (B.D.H.), hexamethonium chloride (Geigy), tubocurarine chloride and dimethyltubocurarine bromide (Borroughs Wellcome) were used. For measurement of the depolarizing action of acetylcholine, the preparation was bathed in frog Ringer solution containing 3×10^{-6} neostigmine.

RESULTS

Effects on depolarization produced by carbachol or acetylcholine

Dose-depolarization curves for both acetylcholine and carbachol were first constructed for the responses of the isolated toe muscle preparation. The shapes of these curves (and the modifications of these curves by the centrally acting drugs) were so closely similar, even though the dose ranges of the two agonists were different and though it was essential to use neostigmine to maintain the acetylcholine depolarization, that it was decided to use carbachol instead of acetylcholine to induce depolarizations in these experiments, and thus avoid any possible influence that neostigmine might have exerted.

The concentrations of paraldehyde, methylpentynol or mephenesin which just produced complete block (the "just-blocking" concentration) to maximal nerve stimulation were found for each preparation. In the presence of these concentrations, each of these centrally active drugs produced similar effects upon the carbachol dose-depolarization curve (Fig. 1), the depression of the depolarization being particularly marked with the

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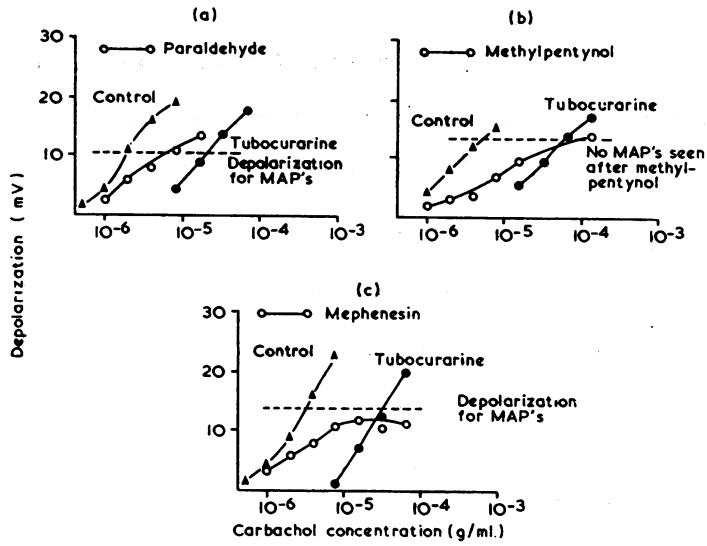

Fig. 1. M. extensor longus digiti IV. Carbachol dose-deporalization curves from three different preparations in the absence and presence of: *a*, paraldehyde 1.4×10^{-3} tubocurarine 2.25×10^{-6}; *b*, methylpentynol 1.75×10^{-3}, tubocurarine 3×10^{-6}; *c*, mephenesin 5.6×10^{-4}, tubocurarine 2.5×10^{-6}. Responses above the dotted line initiated action potentials but not after methylpentynol. ▲=carbachol alone; ○ and ●=carbachol in the presence of the blocking dose of centrally acting drug and tubocurarine respectively.

higher concentrations. With concentrations producing incomplete block to nerve stimulation smaller but similar depressions of the curves were seen.

The records after paraldehyde showed that no change in the extent of the depolarization necessary to initiate action potentials had been produced. After methylpentynol or mephenesin it was often impossible to produce a degree of depolarization similar to that which was necessary to initiate action potentials in the control responses. In those experiments in which larger depolarizations could be initiated, action potentials were initiated in some but not in others.

In the presence of a " just-blocking " concentration of hexamethonium, the shift to the right of the dose-response curve was typical of a competitive blocking action, both the control and the hexamethonium curves being parallel. This shift was, however, consistently greater than that produced by an equi-blocking concentration of tubocurarine (Fig. 2,*a*). In preparations just blocked by dimethyltubocurarine the dose-depolarization response curve was not only shifted to the right but was flattened (Fig. 2,*b*) suggesting a non-competitive anti-carbachol action. Increasing partial-blocking concentrations of dimethyltubocurarine causing increasing degrees of block produced increasing shift and flattening of the dose depolarization-response curves. No change in the threshold level of depolarization necessary to initiate action potentials after dimethyltubocurarine was detected but, in the instance of hexamethonium, no action potentials were seen after the first 5 sec following addition of the agonist. The reason for this appeared to be the rapid accommodation that took place with the high doses of carbachol or acetylcholine

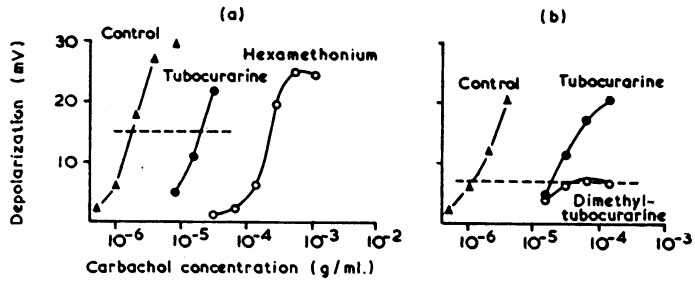

Fig. 2. M. extensor longus digiti IV. Carbachol dose-depolarization curves from two different preparations in the absence and presence of: *a*, hexamethonium 1.2×10^{-3}, tubocurarine 3×10^{-6}; *b*, dimethyltubocurarine 7.5×10^{-5}, tubocurarine 4×10^{-6}. Responses above the dotted line initiated action potentials except in the presence of hexamethonium. ▲=control depolarizations; ● and ○=depolarizations in the presence of tubocurarine and test-drug respectively.

that were necessary to bring about the larger levels of depolarization. If a rapid sweep of the fluid electrode was made as soon as possible after the addition of the agonist, action potentials following these high concentrations could be detected, but it was not possible under these conditions to ascertain whether the threshold of depolarization to initiate muscle action potentials had changed.

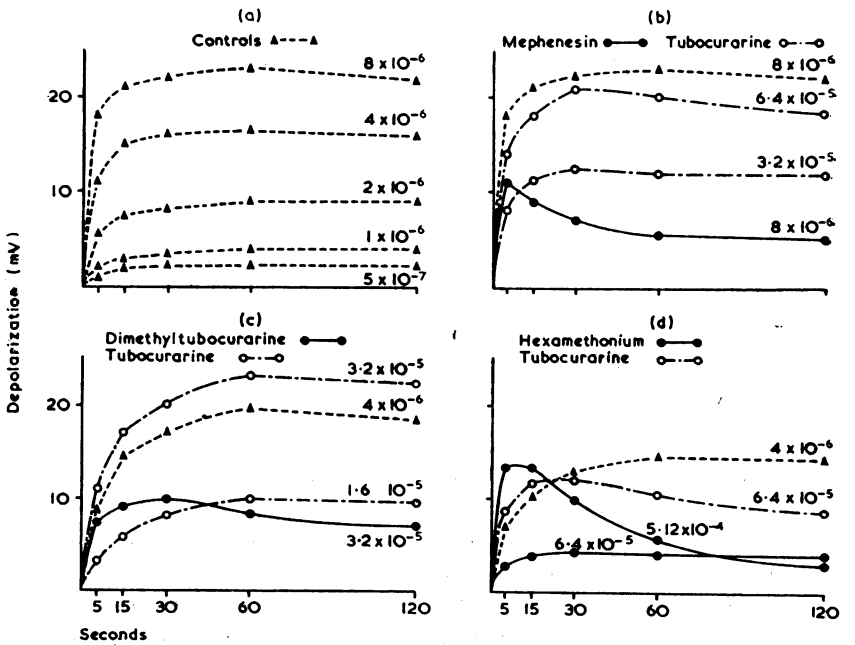

Fig. 3. M. extensor longus digiti IV. The development and maintenance of depolarization to varying doses of carbachol (concentration shown above each curve). ▲=in the absence of any blocking drug: ○=in the presence of *b*, mephenesin 5.6×10^{-4} and tubocurarine 2.5×10^{-6}; *c*, dimethyltubocurarine 5×10^{-5} and tubocurarine 3×10^{-6}; *d*, hexamethonium 5×10^{-4} and tubocurarine 1.5×10^{-6}.

The dose-depolarization curves in Fig. 3 were obtained from a series of depolarization records made 5, 15, 30, 60 and 120 sec after the addition of the agonist to the bath. In the absence of a blocking drug, depolarization was well maintained with all concentrations of carbachol used over the 120 sec period, usually becoming maximal at 30 to 60 sec (Fig. 3,*a*). In the presence of tubocurarine, the depolarization to added agonist was still well maintained over the two-minute period but some decline could be detected at the higher levels of depolarization (Fig. 3,*b*, *c* and *d*). Control experiments with carbachol alone in the concentration range necessary to produce depolarization in the presence of tubocurarine showed a similar slight decline of the depolarization with time. A very marked decline of the depolarization with time occurred when using the very high range of carbachol concentrations similar to those found necessary to depolarize the muscle after hexamethonium. This marked decay of the depolarization could also be seen in the presence of hexamethonium with the higher concentrations of carbachol used (5.2×10^{-4} in Fig. 3,*d*). Flattening of the dose-response curves after mephenesin, methylpentynol, paraldehyde and dimethyltubocurarine was associated with slow development of depolarization with time and was seen after concentrations of agonist which, either alone or in the presence of tubocurarine, did not delay development of depolarization. The curves in Fig. 3,*b*, *c* and *d* permit comparison of the development and maintenance of the depolarization following agonist alone, in the presence of tubocurarine or after the test drug at similar levels of depolarization. The responses obtained after paraldehyde or methylpentynol were similar to those after mephenesin (Fig. 3,*b*).

The mean concentrations of these drugs found necessary to just-block of the M. extensor longus digiti IV are shown with their standard errors in Table 1.

TABLE 1

MEAN DRUG CONCENTRATIONS (WITH STANDARD ERRORS) FOUND NECESSARY TO BLOCK NEUROMUSCULAR TRANSMISSION IN THE ISOLATED M. EXTENSOR LONGUS DIGITI IV OF THE FROG

Drug	Blocking concentration (g/ml.)	m-mole	*n*
Tubocurarine	$3{\cdot}3 \pm 0{\cdot}2 \times 10^{-6}$	0·0048	56
Dimethyltubocurarine	$5{\cdot}2 \pm 0{\cdot}6 \times 10^{-5}$	0·059	11
Mephenesin	$5{\cdot}2 \pm 0{\cdot}3 \times 10^{-4}$	2·86	10
Hexamethonium	$8{\cdot}3 \pm 0{\cdot}6 \times 10^{-4}$	3·04	19
Paraldehyde	$2{\cdot}3 \pm 0{\cdot}2 \times 10^{-3}$	17·4	13
Methylpentynol	$1{\cdot}9 \pm 0{\cdot}1 \times 10^{-3}$	19·4	15

Depression of minature end-plate potentials. All the drugs in concentrations well below those producing complete neuromuscular block reduced the amplitude, with eventual abolition, of the miniature end-plate potentials in the isolated frog sartorius preparation, so providing further evidence of a post-synaptic anti-acetylcholine action. No gross change of frequency of these potentials was detected in the sartorius preparations showing some reduction in the amplitudes of the miniature end-plate potentials. At concentrations nearer to the blocking dose the frequency could not be determined as the amplitude of the miniature end-plate potentials became so reduced as to make it impossible to separate the signal from the base line noise.

Effects on nerve conduction and response to direct muscle stimulation. Of the three centrally active drugs, only mephenesin consistently reduced the size of the nerve action

potential in isolated sciatic nerve. Reductions of 17 to 60% were recorded on exposure to a concentration of 5×10^{-4} g/ml. of mephenesin. Doubling the concentration reduced the action potential still further but good recovery was obtained.

No consistent effects on the muscle action potential in response to direct stimulation were detected in the presence of blocking concentrations of these drugs.

Variations in end-plate potentials. The effects upon amplitude and time course of the end-plate potentials in the presence of just-blocking concentrations of the various drugs differed as is seen in Fig. 4. All records were made from the same M. extensor longus digiti IV preparation. Mephenesin, methylpentynol and paraldehyde revealed end-plate potentials with much greater amplitudes and longer time courses than those seen after tubocurarine. The end-plate potentials following hexamethonium or dimethyltubocurarine were similar to those seen after tubocurarine in the same preparation. As the results in all experiments were not as definite as those illustrated in Fig. 4 the

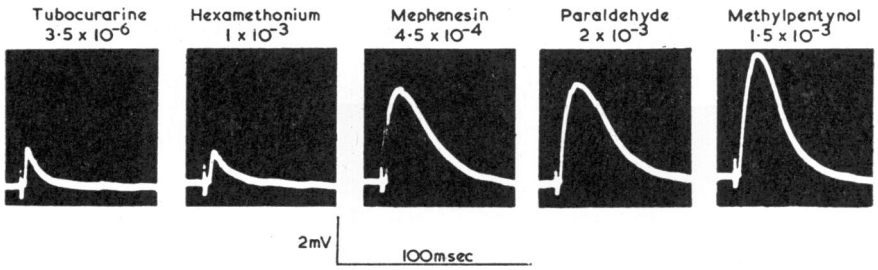

Fig. 4. M. extensor longus digiti IV. End-plate potentials recorded in the same preparation in the presence of blocking concentrations of the drugs named.

differences observed from tubocurarine in the same preparation were subjected to statistical analysis. The parameters measured were peak amplitude (measured to 0.1 mV), time from onset to peak of rise and time from onset to half decay (measured to the nearest msec.). The differences in rise times and times to half decay in the same preparation when compared with tubocurarine were highly significant, P with each drug being <0.001 for methylpentynol ($n=12$), paraldehyde ($n=13$) and mephenesin ($n=10$). Comparison of the mean amplitudes of the mephenesin, methylpentynol and paraldehyde end-plate potentials with the mean amplitude of tubocurarine end-plate potentials recorded in a larger number (59) of different preparations showed these mean values to

TABLE 2

MEAN AMPLITUDES, RISE TIMES AND TIMES TO HALF DECAY OF END-PLATE POTENTIALS IN THE ISOLATED TOE MUSCLE OF THE FROG

Drug	Amplitude (mV)	Rise time (msec)	Time to half decay (msec)	n
Tubocurarine	1·5±0·1	2·3±0·1	8·7±0·6	59
Dimethyltubocurarine	1·0±0·1	1·7±0·2	7·1±1·0	11
Hexamethonium	1·1±0·1	3·3±0·5	11·0±1·2	19
Mephenesin	3·7±0·4	14·1±2·0	42·7±5·5	10
Paraldehyde	2·7±0·5	12·7±1·2	33·3±2·7	13
Methylpentynol	2·8±0·5	11·3±1·2	30·6±2·3	12

be significantly greater after each of the three centrally active drugs ($P<0.001$). The mean values and their standard errors for the end-plate potential amplitudes rise times, and times to half decay for all five blocking drugs are summarized in Table 2.

Effect of neostigmine on neuromuscular block. Only the blockade produced by tubocurarine and dimethyltubocurarine was clearly reversed by soaking the blocked M. extensor longus digiti IV in 3×10^{-6} neostigmine for half an hour. It was then necessary approximately to double the concentration of these blocking drugs to restore block. The time courses of all end-plate potentials, including those after drugs whose actions were not reversed by neostigmine, were prolonged by the anticholinesterase; this

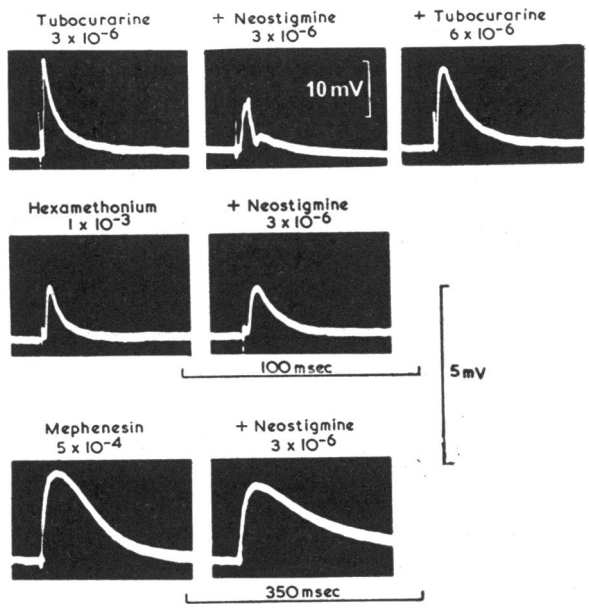

Fig. 5. M. extensor longus digiti IV. The effect of neostigmine on tubocurarine, hexamethonium and mephenesin block. The left-hand records show end-plate potentials at just-blocking concentrations, the centre column shows the response after adding neostigmine. Top right, additional tubocurarine restores block. Note the separate voltage calibration for the muscle action potential in top centre record and the different time base for the lower records.

effect on hexamethonium and mephenesin block together with a comparison of the reversal of a tubocurarine block are illustrated in Fig. 5.

DISCUSSION

The effect of the drugs on the depolarizing activity of carbachol gives a good indication of any specific antagonism to acetylcholine. As judged by the dose-depolarization response curves, only hexamethonium and tubocurarine produced a typical shift of the curve such as would be expected, from a competitive mechanism of action (Clark, 1926; Gaddum, 1937) as typified by tubocurarine. The greater degree of shift of the dose-response curve with hexamethonium than with tubocurarine could be explained by

a greater range of sensitivity of the individual end-plates to the blocking drug hexamethonium. Relatively larger concentrations of hexamethonium as antagonists would then be necessary to produce a 100% block and this in turn would give rise to an apparent difference in sensitivity to added agonist when compared with tubocurarine.

Dimethyltubocurarine shifted the dose-response curve to the right but the flattening of the curve indicated a post-synaptic depressant action of a non-competitive nature.

The results obtained with methylpentynol and paraldehyde differ slightly from those described by Nicholls and Quilliam (1956) in that there was a consistent depression of the depolarization response to the higher doses of agonist in the presence of these drugs, but agree in that there was no marked shift of the curve and no effect on nerve conduction. The reduction in amplitude of the miniature end-plate potentials supports the view that there is a component of post-synaptic depression in the action of these two drugs. Nicholls and Quilliam (1956) suggested that methylpentynol and paraldehyde exerted their blocking action by a pre-synaptic effect altering the release of acetylcholine to nerve stimulation, and such an action is not excluded by my results. In all but its effects on nerve conduction, the responses seen after mephenesin resembled those found after methylpentynol or paraldehyde. A block of conduction in the nerve trunk or presynaptic nerve terminals could account for a decrease in acetylcholine release. In this respect, it is relevant that non-myelinated nerve endings are usually more sensitive to drug action than myelinated nerve trunks (Paintal, 1964); the effect on nerve conduction observed after mephenesin would, therefore, support a presynaptic action. Berger (1947) and Berger and Bradley (1946) have also described a local anaesthetic action of mephenesin. Direct measurements of acetylcholine release in the cat superior cervical ganglion and the rat diaphragm preparations after methylpentynol and paraldehyde have been made by Matthews and Quilliam (1964) and they have shown that, although a decrease in transmitter release does occur after these two drugs, a post-synaptic depression of the effector cell excitability was also indicated.

The time course of maintenance and decay of depolarization after added agonist provides additional information to account for the flattening of the dose-response curves seen after mephenesin, methylpentynol, paraldehyde or dimethyltubocurarine. The desensitization that is seen to added agonists (Thesleff, 1955, 1959; del Castillo and Katz, 1957b; Katz and Thesleff, 1957b) has led to a number of hypotheses involving different rates of association or dissociation of drug-receptor complexes to explain this effect (Katz and Thesleff, 1957b; Paton, 1961). The increased rate of desensitization seen in my experiments after methylpentynol, mephenesin, paraldehyde or dimethyltubocurarine can be explained in terms of alterations in the rates of association or dissociation of the agonist with the receptors. Any such change might be expected to be more marked at the junctional or intrinsic receptors described by Miledi (1960) and del Castillo and Katz (1957a), for they are subjected to recurrent activation by the spontaneously released acetylcholine that gives rise to the miniature end-plate potentials. The extent to which the same receptors are responsible for the generation of end-plate potentials and extrinsically evoked acetylcholine or carbachol potentials has been studied by Goldsmith (1963). His results led him to conclusions similar to those of del Castillo and Katz (1957a) regarding the qualitative pharmacological properties of the two types of receptors. Nevertheless his results did

show quantitative differences both in the rate at which drugs acted and in the magnitude of their effects on acetylcholine potentials and end-plate potentials. It would thus be possible to account for the block of transmission at the neuromuscular junction after mephenesin, methylpentynol or paraldehyde by a post-synaptic action involving an increased rate of desensitization to acetylcholine.

The different amplitudes and time courses of the end-plate potentials seen after mephenesin, methylpentynol or paraldehyde are also indicative of a post-synaptic action. An increase in the resistance of the post-synaptic membrane could account for the production of larger end-plate potentials (Katz & Thesleff, 1957a) and also the increased time course, but Thesleff (1956) found no such change in frog muscle except at concentrations much higher than I used. An anticholinesterase action as an explanation of the long time course of these end-plate potentials is unlikely, as the time course is much longer than that seen after tubocurarine plus neostigmine and the time courses observed can be further prolonged by neostigmine. If the block by mephenesin, methylpentynol or paraldehyde were predominantly pre-synaptic and due to a depression of acetylcholine release, neostigmine would be expected to antagonize the blocking action as the acetylcholine released would be potentiated. The absence of such a reversal in my experiments suggests a post-synaptic action. The inability of neostigmine to reverse the effects of hexamethonium may be related to the suggested differing sensitivities of the individual fibres and the consequent large blocking dose necessary, although a reversal of a partial neuromuscular block in the isolated rat diaphragm preparation treated with hexamethonium has been reported by Deacock & Davies (1958).

The use of the frog neuromuscular junction in assessing the mechanism of action of a drug such as hexamethonium, the competitive action of which is predominantly at ganglionic synapses, can be justified by the fact that the transmitter released at the two synapses is identical and by the observation of a similar competitive mechanism of action at the junction. Any inference as to the mechanism of action of centrally active drugs is, of course, highly speculative but it is of interest that the suggestions that mephenesin, methylpentynol or paraldehyde act at the neuromuscular junction by mechanisms involving changes in desensitization of receptors and/or transmitter release, might also apply to synapses where different transmitters are involved.

SUMMARY

1. The actions of mephenesin, methylpentynol, paraldehyde, hexamethonium and dimethyltubocurarine on the frog neuromuscular junction was investigated using extra- and intra-cellular electrical recording techniques.

2. At neuromuscular blocking concentrations of hexamethonium, a competitive type of dose-depolarization curve was found.

3. The carbachol dose-depolarization curve in the presence of dimethyltubocurarine showed evidence of a non-competitive component in its antagonism to carbachol.

4. In the presence of mephenesin, methylpentynol or paraldehyde, the dose-deporalization curves suggested a non-competitive anti-carbachol and anti-acetylcholine action.

5. The non-competitive actions observed were associated with a definite decline of the depolarization with time.

6. Further evidence in support of a post-synaptic action of methylpentynol, paraldehyde and mephenesin is derived from differences in the end-plate potentials recorded.

7. The post-synaptic action of the centrally active drugs is discussed in relation to current theories of acetylcholine desensitization.

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