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A COMPARISON OF THE EFFECTS OF SOME ETHONIUM IONS AND THEIR STRUCTURAL ANALOGUES ON NEUROMUSCULAR TRANSMISSION IN THE CAT

BY

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In recent years the effects of ethonium ions on neuromuscular transmission have been studied with a variety of experimental techniques. Interest in these effects derives from the fact that some ethonium ions are potent anticurare and contractile-potentiating agents although they have very weak anticholinesterase activity. An important finding in this area of research was that the facilitatory ion triethyl(3-hydroxyphenyl)ammonium (3-OH-PTEA) affects the motor axon terminals, thereby causing antidromic repetitive discharges in the motor nerve following a single orthodromic impulse (Riker, Roberts, Standaert & Fujimori, 1957; Riker, Werner, Roberts & Kuperman, 1959a,b). On the basis of different types of experimental results, a presynaptic locus has also been postulated for the facilitatory action of tetraethylammonium at the amphibian (Koketsu, 1958) and mammalian (Stovner, 1958) neuromuscular junctions. In a crustacean nerve-muscle preparation, 3-OH-PTEA, triethylphenylammonium (PTEA) and tetraethylammonium (TEA) were found to potentiate neurally-evoked contractions, and the evidence again suggested a presynaptic site of action (Kuperman, 1963).

Assuming that the facilitatory actions of ethonium ions on neuromuscular transmission are associated with presynaptic effects in mammalian, amphibian and crustacean organisms, several questions remained to be answered. For example, is the mechanism of the presynaptic effect the same for all ethonium ions ? Do all ethonium ions have equal maximum facilitatory effects on neuromuscular transmission ? Do ethonium ions have only facilitatory effects on neuromuscular transmission, or do they also have depressant actions ? The present investigation is an attempt to answer these and related questions. For this purpose, the effects of several ethonium ions on a mammalian nerve-muscle preparation were determined under various experimental conditions, and structure-activity relationships were examined. The results indicate that ethonium ions cannot be viewed as a homogeneous class of pharmacologic agents with respect to their actions at the mammalian neuromuscular junction.

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METHODS

Neurally-evoked muscle contraction. All experiments were performed on cats anaesthetized with α-chloralose (80 mg/kg, intravenously). The sciatic nerve was cut in the gluteal region, and dissection of the popliteal fossa was carried out as previously described by Riker *et al.* (1959b). In brief, all branches of the popliteal artery except those supplying the gastrocnemius muscle were ligated. The Achilles tendon was connected by heavy steel wire to a strain gauge (Grass Instrument Co., FT-10) the output of which was connected to a carrier amplifier and ink-writer. The whole leg was rigidly fixed in a horizontal position to a myograph stand, and the muscle was adjusted to resting length. Supramaximal rectangular wave pulses of 0.5 msec duration were applied to the popliteal nerve once every 5 sec through bipolar platinum electrodes. The exposed tissues were immersed under a pool of mineral oil maintained at 38° C by a thermoregulator.

Ventral root action potentials. Cats were anaesthetized with chloralose and the spinal cord was cut at T12. The popliteal fossa was exposed and prepared as described above. The lumbar vertebrae were then exposed and a dorsal laminectomy was performed. The procedure was essentially as described by Werner (1960a,b). The popliteal area was bathed in mineral oil, and the lumbar area was bathed in a mineral oil-Krebs-Ringer mixture which was continuously bubbled with 95% oxygen and 5% carbon dioxide. The temperature of both pools was maintained at 38° C. Homolateral to the dissected popliteal fossa, the ventral roots of L7 and S1 were disconnected from the spinal cord. At least five filaments were isolated from these ventral roots, each filament containing one or very few active axons. Rectangular wave pulses of 0.1 msec duration were applied once every 7 sec to the ventral root filaments via bipolar platinum electrodes; the stimulus voltage was adjusted so that contraction of gastrocnemius muscle fibres could be observed. A monopolar platinum recording electrode was placed about 15 mm distal to the stimulating electrode. Monophasic action potentials were amplified, displayed on a cathode-ray oscilloscope, and photographed.

Denervated muscle. Under aseptic conditions and pentobarbitone sodium anaesthesia, the sciatic nerve was cut in the gluteal region. After 10 to 14 days the popliteal fossa was dissected and the gastrocnemius muscle was prepared for recording contraction as described above. The muscle-stimulating electrode was a 20-gauge needle inserted into the belly and a metal clip was applied to the musculotendinous junction. Rectangular wave pulses of 2 msec duration and maximal voltage were applied through these electrodes once every 5 sec.

Materials. All drugs were dissolved in mammalian saline, and the concentration of the solution was such that the dose per kg was contained in 0.05 ml. All injections were made directly into the popliteal artery using a syringe fitted with a 27-gauge needle. The monoquaternary ammonium ions were synthesized by Eastman Kodak. Benzoquinonium was obtained through the courtesy of Sterling-Winthrop and crystalline tubocurarine was obtained from Mann (U.S.A.). A list of compounds used in this investigation, and their notations, is given in Table 1.

TABLE 1

LIST OF COMPOUNDS AND THEIR NOTATIONS

All compounds are the iodides, except where indicated otherwise

Compound	Notation
Triethyl(3-hydroxyphenyl)ammonium	3-OH-PTEA
Diethyl(3-hydroxyphenyl)methylammonium	3-OH-PDEMA
Triethyl(4-hydroxyphenyl)ammonium	4-OH-PTEA
Diethyl(4-hydroxyphenyl)methylammonium	4-OH-PDEMA
Triethylphenylammonium	PTEA
Diethylmethylphenylammonium	PDEMA
Tetraethylammonium	TEA
Triethylmethylammonium	TEMA
Triethyl-*m*-tolylammonium	3-CH_3-PTEA
Triethyl(3-methoxyphenyl)ammonium	3-CH_3O-PTEA
Triethyl(3-ethylphenyl)ammonium	3-C_2H_5-PTEA
3-Aminophenyltriethylammonium	3-NH_2-PTEA
Triethyl(3-nitrophenyl)ammonium	3-NO_2-PTEA
(+)-Tubocurarine chloride	Tubocurarine
Benzoquinonium chloride	Benzoquinonium

RESULTS

Effects of ethonium ions on neurally-evoked contractile response : dose-response relationships

The relationship between dose and contractile potentiation is shown for three ethonium ions in Fig. 1. Within the dose range shown for each ion, the calculated regression lines are significantly linear ($P<0.05$). It is obvious that amongst the ethonium ions 3-OH-PTEA, PTEA and TEA, considerable differences exist with respect to maximum potentiating action and regression line slopes. In Table 2, actual values of the maximum responses and

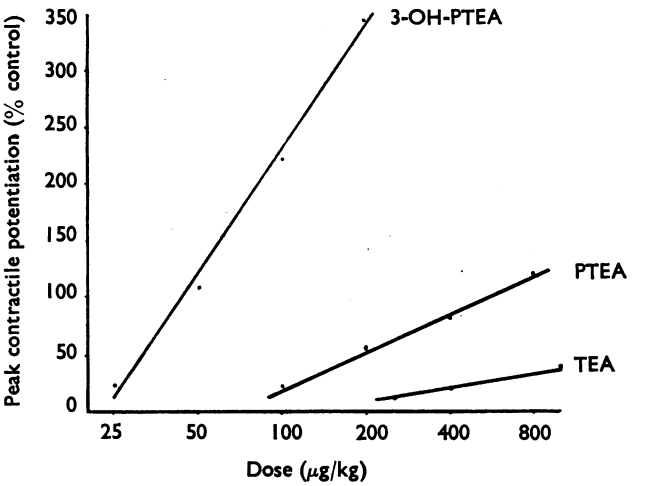

Fig. 1. Relationship between dose (on log scale) and magnitude of contractile potentiation (of neurally-evoked muscle contractions) produced by three ethonium ions. Each point is the average of six independent observations.

TABLE 2

SOME COMPARISONS BETWEEN EFFECTS OF QUATERNARY AMMONIUM IONS ON THE NEURALLY-EVOKED CONTRACTILE RESPONSE

The maximum potentiating dose of each compound was estimated on six preparations. Increasing doses above values shown produced decreasing degrees of potentiation and eventually depression. The slope (b) of each regression line was calculated from three or four mean points and standard errors on the linear portion of the dose/response curve. S_b (%) is the standard error of the slope expressed as a percentage of b. The *t*-test was used to determine significant differences between slopes. Within each group of compounds, no significant differences were found between slopes ($P>0.05$). The slope of any compound in Group I was significantly greater than that of any other compound ($P<0.05$). The slope of either Group II compound was significantly greater than that of either Group III compound ($P<0.05$)

Compound	Maximum potentiating dose (μg/kg)	Mean maximum response (% of control)	b	S_b (%)
Group I				
3-OH-PDEMA	60	402	354·82	10·00
3-OH-PTEA	300	383	347·75	7·18
4-OH-PDEMA	500	370	300·17	4·04
4-OH-PTEA	800	362	306·87	1·12
Group II				
PDEMA	800	181	122·56	6·15
PTEA	1,000	176	104·78	8·28
Group III				
TEMA	1,000	36	26·58	11·62
TEA	1,000	38	20·10	5·88

regression line slopes are listed for these and other quaternary ammonium ions which potentiate neurally-evoked contraction. Significant differences in threshold potencies have been previously reported, and threshold values are not reproduced here (Kuperman, Gill & Riker, 1961).

It is worth noting that the maximum potentiating effects of 3-OH-PTEA and other hydroxyanilinium ions were minimized in earlier investigations (see Riker *et al.*, 1957; Kuperman *et al.*, 1961) by the poor dynamic properties of the mechanical recording system.

Representative tracings of the contractile-potentiating actions of the ethonium ions are shown in Fig. 2. Each tracing shows that the first contraction following drug injection was

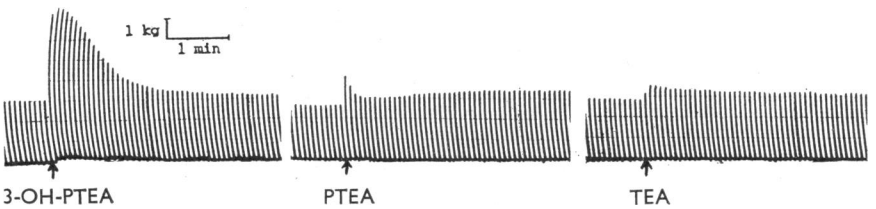

Fig. 2. Typical potentiating responses (of neurally-evoked contractile responses) to 3-OH-PTEA (50 μg/kg), PTEA (200 μg/kg) and TEA (500 μg/kg). Note biphasic potentiation produced by PTEA. Calibrations, 1 kg and 1 min.

potentiated, and peak potentiation occurred either at the first or second post-injection contraction. With larger doses of 3-OH-PTEA, the peak effect might require the passage of up to six contractions (top tracing in Fig. 3, for example). However, the first contraction after drug injection was always potentiated, and this type of time-action curve differs considerably from that associated with typical anticholinesterase agents like physostigmine; with such agents there is always a latent period between drug administration and the first sign of potentiation regardless of dose.

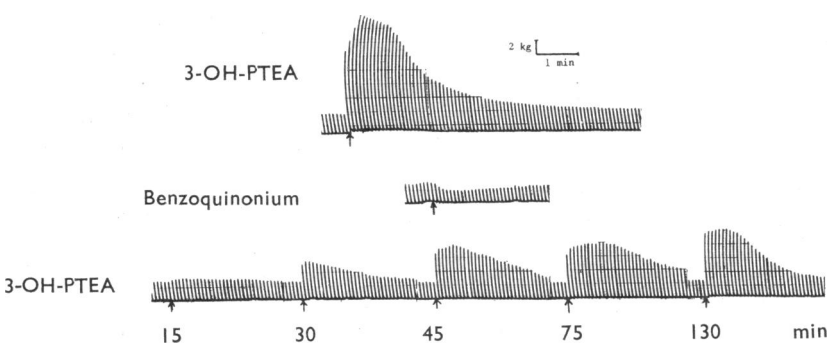

Fig. 3. Depression of response to 3-OH-PTEA after benzoquinonium (on neurally-evoked contractile responses). Injection of benzoquinonium (10 μg/kg) was made 15 min after that of 3-OH-PTEA. Each arrow under the bottom tracing indicates an injection of 3-OH-PTEA. All injections of 3-OH-PTEA were of 200 μg/kg and at the stated period of time after benzoquinonium. Calibrations, 2 kg and 1 min.

The peculiar biphasic response produced by PTEA in doses ranging from 100 to 800 μg/kg (Fig. 2) deserves comment. Although the magnitude of the first response varied according to dose up to the ceiling indicated in Fig. 1, the magnitude of the second response varied only between 19 and 33% of control over the range of doses studied. The total time from the beginning of the first sign of potentiation to the complete decay of the second potentiating response was 11 min (mean of eighteen observations). Similarly, the maximum potentiation produced by TEA was 22 to 38% of control, and the total duration of effect was 9 min (mean of twenty-six observations). In contrast to the relatively long duration of action of TEA or PTEA, the effect of 3-OH-PTEA subsided within 3 to 5 min despite the greater magnitude of response.

Effect of benzoquinonium on ethonium-ion response

In doses having no effect on contraction, benzoquinonium blocks the potentiating action of edrophonium (the *N*-dimethylethyl analogue of 3-OH-PTEA) (Bowman, 1958). Moreover, whereas a weak potentiator like TEA is an effective antagonist of paralysis due to benzoquinonium, a potent potentiating ion like edrophonium is virtually without effect (Blaber & Bowman, 1959). These experimental findings suggested that the ethonium ions under present consideration be studied in conjunction with benzoquinonium in order to establish additional criteria for differentiating between the pharmacologic properties of these ions.

In each experiment, a certain dose of ethonium ion was injected before and after administration of benzoquinonium. In order to have a basis for comparison, the ethonium ion was always injected exactly 15 min after benzoquinonium, and subsequent injections were repeated at 15- to 25-min intervals until the magnitude of pharmacologic response returned to at least two-thirds of control level. In separate experiments, it was shown that, in the absence of benzoquinonium, any of the ethonium ions could be injected at 15-min intervals with little or no changes in response magnitude.

The doses of benzoquinonium selected for these experiments were 5 and 10 μg/kg. The dose of 5 μg/kg produced only a 4% depression of contraction amplitude (mean of twenty-eight observations) which lasted for 1 min or less. In over half of the preparations tested, this dose of benzoquinonium had absolutely no effect on contraction. At 10 μg/kg, benzoquinonium produced a 38% depression of contraction amplitude (mean of sixty-one observations) which decayed within 2 to 12 min.

3-OH-PTEA. The results of a typical experiment with 3-OH-PTEA and benzoquinonium are illustrated in Fig. 3. In this example, maximum potentiating doses of the ethonium ion were injected before and after 10 μg/kg of benzoquinonium; note the almost total abolition of ethonium-ion response 15 min after benzoquinonium. After an additional 1.5 hr (not shown in Fig. 3), the potentiating response returned to within 10% of control level. The results of five other experiments, using the same doses of 3-OH-PTEA and benzoquinonium, were similar. The times to two-thirds recovery of the potentiating responses to various doses of 3-OH-PTEA after treatment with benzoquinonium are shown in Table 3; note the inverse relationship between recovery time and dose of 3-OH-PTEA. For example, the potentiation produced by 50 μg/kg of 3-OH-PTEA 3 hr after 10 μg/kg of benzoquinonium was only 65% of control (mean of five observations). With larger doses of 3-OH-PTEA, complete recovery of normal pharmacologic response occurred within 2 hr.

TABLE 3

RECOVERY OF RESPONSE TO 3-OH-PTEA AFTER INJECTION OF BENZOQUINONIUM OR TUBOCURARINE

Injections of 3-OH-PTEA were made at 15- to 25-min intervals and recovery curves were plotted. Each point on a curve was the average of five independent observations calculated as (percentage increase in contraction amplitude after benzoquinonium or tubocurarine/percentage increase in contraction amplitude before benzoquinonium or tubocurarine) × 100. Recovery times are times to two-thirds of control potentiating response to 3-OH-PTEA

Dose of 3-OH-PTEA (μg/kg)	Recovery time (min) after Benzoquinonium 5 μg/kg	Benzoquinonium 10 μg/kg	Tubocurarine 20 μg/kg
50	88	173	129
100	50	81	86
200	34	52	44

At 5 μg/kg, benzoquinonium also antagonized the potentiating action of 3-OH-PTEA, but this antagonism was neither as complete nor as enduring as that produced by 10 μg/kg (Table 3). For example, the average response to 200 μg/kg of 3-OH-PTEA just 15 min after 5 μg/kg of benzoquinonium was 46% of that normally obtained (five experiments); the response to ethonium ion returned to normal within another 30 to 45 min. Analogous results were obtained using smaller doses of 3-OH-PTEA in conjunction with 5 μg/kg of benzoquinonium. In each instance the degree of depression of the ethonium-ion response and the time to recovery were less than observed after 10 μg/kg of benzoquinonium. Nevertheless, even this relatively limited action of benzoquinonium is interesting because in over 50% of the preparations the 5-μg/kg dose produced no depression of contraction amplitude; it was only after challenging the preparation with 3-OH-PTEA that an effect of this dose of benzoquinonium became evident in these cases.

The response to 3-OH-PTEA after treatment with benzoquinonium frequently consisted of depression rather than potentiation of contraction (Fig. 4). At 15 min after the injection

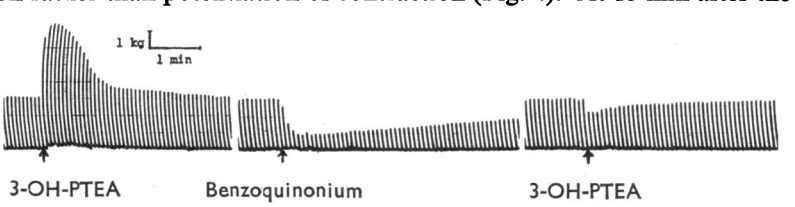

Fig. 4. Reversal of response to 3-OH-PTEA after benzoquinonium (on neurally-evoked contractile response). Injection of benzoquinonium (10 μg/kg) was made 15 min after that of 3-OH-PTEA. The tracing on the far right shows the response to 3-OH-PTEA 15 min after benzoquinonium; both injections of 3-OH-PTEA were of 100 μg/kg. Calibrations, 1 kg and 1 min.

of 10 μg/kg of benzoquinonium, 50 μg/kg of 3-OH-PTEA produced depression in 40% of the experiments; the average magnitude of this depression was 32%. At 30 min after benzoquinonium, the same dose of 3-OH-PTEA caused depression in 15% of the experiments, the average magnitude being 18%. This " reversal " of pharmacologic response to 50 μg/kg of 3-OH-PTEA was also observed after administration of 5 μg/kg of benzoquinonium. At 100 or 200 μg/kg, 3-OH-PTEA always caused potentiation after treatment with benzoquinonium but, as described above, this potentiation was less than that obtained in the absence of benzoquinonium; these responses probably represented algebraic summation of potentiating and depressant actions on neuromuscular transmission.

TEA. In contrast to the marked antagonism of 3-OH-PTEA potentiation by benzoquinonium, this compound produced no reduction of TEA potentiation with respect to either amplitude or time course (Fig. 5). The relationship between dose of TEA and potentiation of contraction amplitude shown in Fig. 1 can be reproduced if the various doses of TEA are given 15 min after 10 or even 20 μg/kg of benzoquinonium.

Other interesting findings emerged from the studies of TEA—benzoquinonium interaction. For example, injection of 500 μg/kg of TEA during the course of a depressant action produced by 10 to 15 μg/kg of benzoquinonium caused not only an increase in contraction amplitude to control level but also about 20 to 30% potentiation (Fig. 6). From the results of the experiments on 3-OH-PTEA—benzoquinonium interaction, described above, a

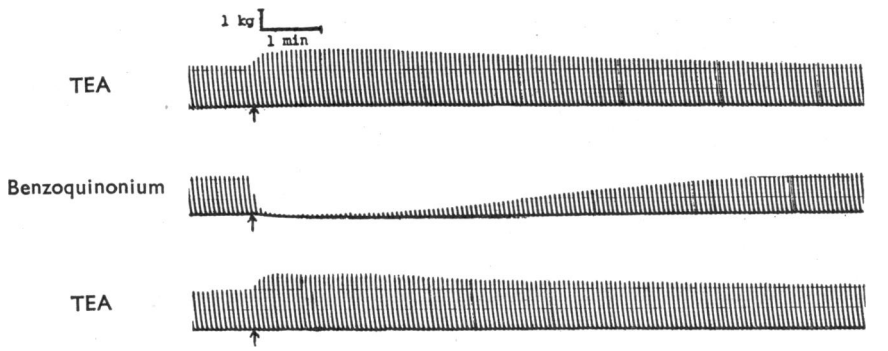

Fig. 5. Lack of effect of benzoquinonium on TEA (500 μg/kg) response (on neurally-evoked contractile response). Injection of benzoquinonium (20 μg/kg) was made 15 min after that of TEA. The bottom tracing shows the response to 500 μg/kg of TEA 15 min after benzoquinonium; the magnitude of this response is the same as that shown in the top tracing. Calibrations, 1 kg and 1 min.

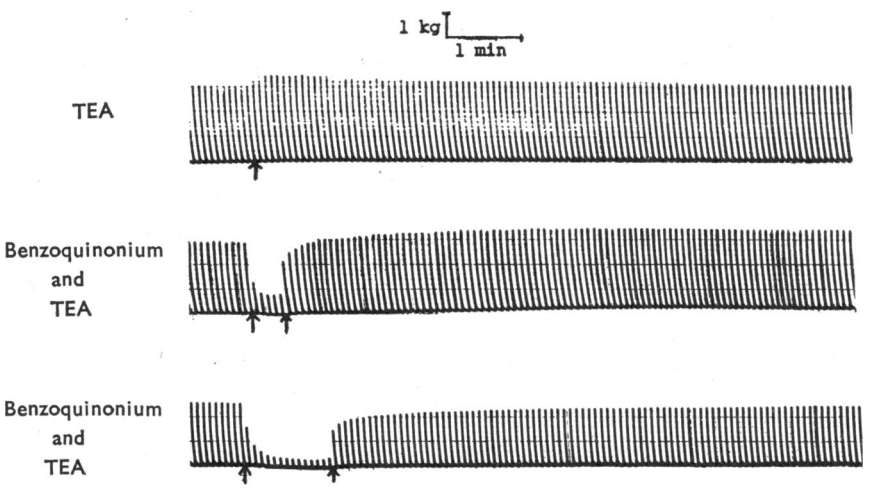

Fig. 6. Antagonism of paralysis due to benzoquinonium (10 μg/kg in the middle record, and 20 μg/kg in the bottom record, at the first arrows) by TEA (on neurally-evoked contractile response). The dose of TEA in each instance is 500 μg/kg, and was given at the second arrow in the lower two records. Note in the middle tracing that TEA produces slight contractile potentiation. Calibrations, 1 kg and 1 min.

similar response to a maximum potentiating dose of 3-OH-PTEA would not be anticipated; in fact, injection of 3-OH-PTEA during the course of benzoquinonium paralysis produced a transient further decrease in contraction amplitude. These results with 3-OH-PTEA are similar to those previously obtained with edrophonium (Bowman, 1958; Blaber & Bowman, 1959) and, therefore, will not be elaborated on here.

The magnitude of antagonism of benzoquinonium paralysis by TEA is dependent, of course, on the doses of both compounds. Injection of a maximum potentiating dose of TEA at the peak of an 80% depression produced by 20 μg/kg of benzoquinonium caused complete antagonism of paralysis, but did not cause potentiation (Fig. 6). Remarkably, 500 μg/kg of TEA reduced even a complete neuromuscular paralysis (40 μg/kg of benzoquinonium) to about 10 to 20% depression within 1 min after injection.

PTEA. The interaction between PTEA and benzoquinonium was complicated, undoubtedly because the PTEA action by itself is biphasic, as previously described. At 15 min after injection of 10 μg/kg of benzoquinonium, the response to PTEA (200 to 800 μg/kg) was biphasic; the initial phase, however, consisted of depression rather than potentiation of contraction and the second phase consisted of potentiation (Fig. 7). The

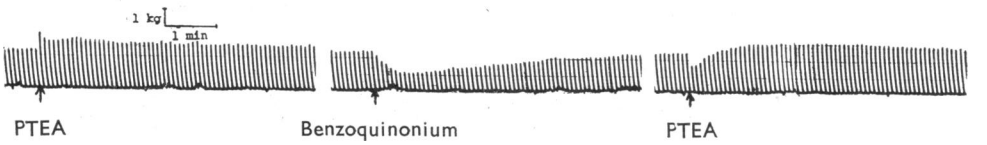

Fig. 7. Effect of benzoquinonium (10 μg/kg) on the response to PTEA (on neurally-evoked contractile response). Injection of benzoquinonium was made 15 min after that of PTEA (200 μg/kg). The right-hand tracing shows the response to 200 μg/kg of PTEA 15 min after benzoquinonium; note the contractile potentiation following depression. Calibrations, 1 kg and 1 min.

magnitude of the depression was 13 to 34% of control, and the potentiation ranged from 14 to 28% (twelve observations). Thus, the initial potentiating response to PTEA was altered by benzoquinonium, and this consisted of a " reversal " of effect already described for 3-OH-PTEA; the second potentiating response appeared to be unaffected. The action of PTEA on a paralysis by benzoquinonium was similar to that of TEA; a 40 to 60% depression was completely antagonized by 500 μg/kg of PTEA and, moreover, a slight potentiation was observed.

Benzoquinonium accumulation. It is noteworthy that the potentiating action of 3-OH-PTEA was reduced or even reversed by benzoquinonium at a time when contraction amplitude was at control level, that is at a level recorded before the benzoquinonium injection. Perhaps there exists a long-lasting depressant action of benzoquinonium on the postsynaptic membrane which somehow limits the potentiating response to 3-OH-PTEA without simultaneously affecting neuromuscular transmission. If so, it should be possible to estimate the duration and intensity of this action by measuring the effects on contraction amplitude of two successive benzoquinonium injections. In each of six preparations, two injections of 5 μg/kg of benzoquinonium were made 15 min apart; in six other preparations this procedure was repeated using the 10-μg/kg dose. At 5 μg/kg, there was no significant difference between the mean-percentage depression of contraction amplitude produced by the two injections ($P>0.2$). At 10 μg/kg, there was a significant difference

($P<0.05$), the percentage depression obtained after the second injection being almost twice as great as that obtained after the first. Despite the lack of evidence for accumulation of the effect of 5 μg/kg of benzoquinonium given at 15-min intervals, it should be stressed that the 3-OH-PTEA potentiation was severely attenuated by this dose of benzoquinonium. Furthermore, the TEA potentiation and the secondary potentiating response to PTEA were unaltered by either 5 or 10 μg/kg of benzoquinonium.

Effect of tubocurarine on response to ethonium ion

In previous experiments (see Bowman, 1958; Blaber & Bowman, 1959) certain pharmacologic differences between benzoquinonium and tubocurarine were emphasized, for example, the relatively weak antagonism by edrophonium of a paralysis by benzoquinonium compared to one by tubocurarine. Because edrophonium is a close structural analogue of 3-OH-PTEA, it was decided to test the interaction between tubocurarine and ethonium ions in the same manner as described above for benzoquinonium. Some experiments along these lines have been previously reported (Kuperman, 1961; Werner & Kuperman, 1963). In brief, the results were qualitatively similar to those obtained with benzoquinonium: the potentiating action of 3-OH-PTEA was reduced, abolished or reversed, depending on the doses of tubocurarine and 3-OH-PTEA used; the biphasic potentiating action of PTEA was converted into another biphasic action consisting of depression followed by potentiation; the TEA potentiation was unaltered. From a quantitative view, it is interesting that, unless doses of tubocurarine which depressed contraction by at least 30 to 40% were used, no modification of ethonium-ion potentiation was observed. A dose of tubocurarine which almost completely abolished or even reversed the 3-OH-PTEA response in five preparations was 20 μg/kg; this effect of tubocurarine was comparable to that produced by 10 μg/kg of benzoquinonium. The results of a typical tubocurarine experiment are illustrated in Fig. 8, and a comparison between the effect of benzoquinonium and tubocurarine on the response to ethonium ion is shown in Table 3.

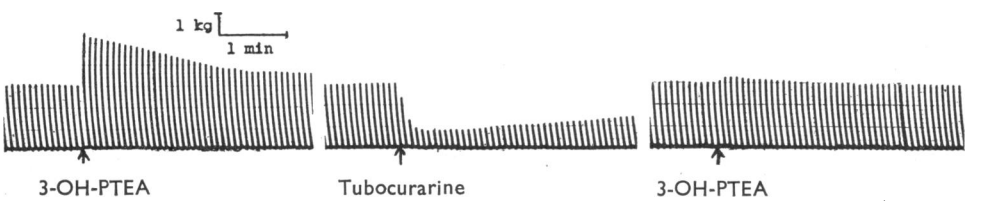

Fig. 8. Depression of response to 3-OH-PTEA after tubocurarine (on neurally-evoked contractile response). Injection of tubocurarine (20 μg/kg) was made 15 min after that of 3-OH-PTEA (100 μg/kg). The right-hand tracing shows the response to 100 μg/kg of 3-OH-PTEA 15 min after tubocurarine. Calibrations, 1 kg and 1 min.

The antagonism by TEA or PTEA of a paralysis by tubocurarine was qualitatively and quantitatively similar to the antagonism of a paralysis due to benzoquinonium. The striking anticurare action of TEA was recognized long ago by Kensler (1950); but it is important to emphasize here that the injection of 500 μg/kg of TEA or PTEA during the course of a 60 to 90% paralysis by tubocurarine, caused an increase of contraction amplitude *above* control level. In contrast, the action of 3-OH-PTEA on this magnitude of paralysis by tubocurarine was very limited. The injection of twice the maximum potentiating dose

of 3-OH-PTEA (400 μg/kg) caused reduction of the depression to about 10 to 15% of control, and did not result in potentiation (six experiments). This relatively incomplete type of tubocurarine antagonism by 3-OH-PTEA has been previously described (Riker *et al.*, 1957).

Structure-activity relationships

The effects of three groups of compounds were determined and compared with those produced by 3-OH-PTEA, PTEA and TEA. These groups are: (1) analogues of the aforementioned ions in which one ethyl group is replaced by a methyl; (2) *p*-hydroxyanilinium ions; and (3) analogues of 3-OH-PTEA in which the *m*-hydroxyl group is replaced by another moiety. Each compound in groups 1 and 2 was found to be similar to its corresponding ethonium-ion analogue with respect to those pharmacologic properties previously described. Thus, 3-OH-PDEMA, 4-OH-PDEMA and 3-OH-PTEA are pharmacologically analogous to 3-OH-PTEA with respect to potentiation of contraction and antagonism by benzoquinonium or tubocurarine. Slope values of the dose-contractile potentiation regression lines of these hydroxyanilinium ions do not differ significantly from each other (Table 2). The compound PDEMA is similar to PTEA with respect to the slope value of its dose-response regression line (Table 2); but, with respect to its antagonism by benzoquinonium or tubocurarine and its curve of potentiating action, it is more similar to the hydroxyanilinium ions. TEMA and TEA are pharmacologically analogous in every respect.

TABLE 4

LIST OF ETHONIUM IONS WHICH PRODUCE ONLY DEPRESSION OF NEUROMUSCULAR TRANSMISSION

The threshold depressant dose is that which produced 15 to 25% depression of contractile amplitude in at least four experiments

Compound	Threshold depressant dose (μg/kg)
3-CH_3-PTEA	30
3-CH_3O-PTEA	100
3-C_2H_5-PTEA	250
3-NH_2-PTEA	500
3-NO_2-PTEA	500

In Table 4, *meta*-substituted triethylphenylammonium ions are listed which lack the capacity to potentiate contraction at any dose. In fact, each of these ethonium ions are depressants of neurally-evoked contraction. Complete dose-response curves were not obtained for these ions because, for purposes of this investigation, it is important to note only that triethylphenylammonium ions have either potentiating or depressant actions on neuromuscular transmission, depending on the nature of the phenyl substituent. Interestingly, the threshold depressant potencies of some of these ethonium ions approach that of tubocurarine. For example, the approximate threshold depressant dose of 3-CH_3-PTEA is only about two- to three-times that of tubocurarine under the conditions of these experiments.

Actions on the motor nerve terminal

Previous experiments have indicated that contractile potentiation by 3-OH-PTEA and other hydroxyanilinium ions is causally related to an action on the motor nerve terminals

(Riker *et al.*, 1957, 1959a,b; Werner, 1960a,b). The prejunctional effect was signalled in these experiments by antidromic repetitive discharges in the motor axon following an orthodromic impulse evoked by a single stimulus.

In the present study of ethonium-ion action on motor nerve terminals, each preparation was set up to record antidromic impulses in five ventral root filaments. All compounds were injected in doses which cause maximum potentiation of contraction as determined in separate dose/response experiments (Fig. 1). A compound was considered to be effective in any given preparation if the antidromic response was recorded from at least one out of the five ventral root filaments sampled. The compound 3-OH-PDEMA was used as a reference standard because it is the most potent contractile-potentiating agent of all the hydroxyanilinium ions tested (Kuperman *et al.*, 1961). The antidromic response was produced by 10 μg/kg of this ion in eight out of fourteen preparations. In each of these eight preparations, the ethonium ions 3-OH-PTEA, PTEA and TEA were injected at 30-min intervals in random order. The results of these experiments are summarized in

TABLE 5

COMPARISON OF THE EFFECTIVENESS OF VARIOUS COMPOUNDS IN PRODUCING REPETITIVE ANTIDROMIC DISCHARGE

Pairs of numbers give the numbers of cats showing antidromic response and the numbers of cats tested. Only those cats which responded to 3–OH–PDEMA were used for testing ethonium ions

Compound	Dose (μg/kg)	Responses to	
		Single stimuli	Paired stimuli
3–OH–PDEMA	10	8/14	—
3–OH–PTEA	200	5/8	8/8
PTEA	800	3/8	8/8
TEA	1,000	0/8	5/8

Table 5, and a typical antidromic response is shown in Fig. 9. Whereas 3-OH-PTEA was effective in over half the preparations tested, TEA was ineffective in all of them. Although PTEA was effective in three preparations, the time course of the response was considerably different from that associated with 3-OH-PTEA. After the injection of 3-OH-PTEA, antidromic impulses were observed for the ensuing 3 to 8 min. In contrast, the PTEA effect was fleeting and appeared only during the two or three oscilloscope sweeps immediately following injection (about 14 to 21 sec).

Each ethonium ion was retested under conditions which increase excitability of the prejunctional terminals, that is in conjunction with paired stimuli at close intervals (Werner,

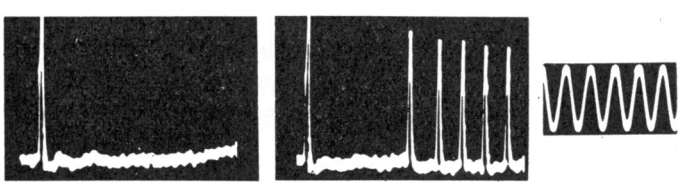

Fig. 9. Antidromic repetitive afterdischarge in a single ventral root fibre after injection of 200 μg/kg of 3-OH-PTEA. Rectangular wave pulse of 0.1 msec duration was applied every 7 sec to the same fibre. Left-hand tracing: control showing orthodromic spike only. Right-hand tracing: 30 sec after drug injection, showing antidromic spikes following single orthodromic spike on left. Time signal, 500 cycles/sec.

1960; Hubbard & Schmidt, 1963). Pulse pairs with a 1.7- to 3-msec interval were applied once every 7 sec to the motor nerve. The results of these experiments are also summarized in Table 5, and typical responses are shown in Fig. 10. In conjunction with paired stimulation, both TEA and PTEA produced antidromic impulses in almost all preparations, and 3-OH-PTEA was consistently effective. Note that the antidromic responses to these ethonium ions are similar with respect to (1) latency between second orthodromic impulse and first antidromic impulse, (2) frequency of antidromic discharge, and (3) number of antidromic impulses following each pair of orthodromic spikes. Surprisingly, even the total duration of the effect is about the same for all compounds.

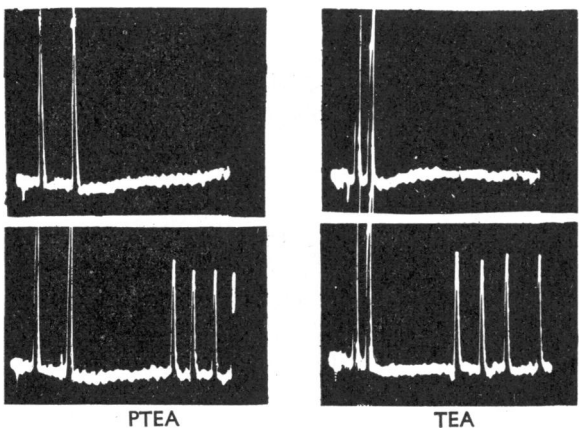

Fig. 10. Antidromic repetitive afterdischarge in a single ventral root fibre after injection of PTEA (200 μg/kg) or TEA (500 μg/kg). Pulse pairs were applied every 7 sec to the same fibre as used for recording. Pulse pair intervals were 2.5 msec and 1.7 msec in the PTEA and TEA experiments respectively. Top tracings: pre-injection controls. Bottom tracings: 1 min after drug injection. The oscilloscope sweep speed same in both experiments.

The potentiating ions 4-OH-PTEA, PDEMA and TEMA were tested in two preparations for their capacity to produce the antidromic response. In the presence of single nerve shocks, each compound was ineffective. In conjunction with close-interval paired stimuli, 4-OH-PTEA and PDEMA produced antidromic discharge in two preparations, and TEMA did so in one.

Two ethonium ions which were found to be neuromuscular depressants, 3-CH_3-PTEA and 3-CH_3O-PTEA, were tested in three additional preparations for signs of prejunctional action. Three doses of each ion were injected: a subdepressant dose, a threshold-depressant dose, and a dose which caused at least a 40% depression of contractile amplitude. Both ions were ineffective in causing the antidromic response at any dose level, even when tested in conjunction with paired nerve stimuli.

Actions on chronically denervated muscle

When injected in doses which cause maximum potentiation of neurally-evoked contraction, the ethonium ions produced contraction of chronically denervated muscle. An example of the contractile response to TEA is illustrated in Fig. 11. Note that the magnitude of this

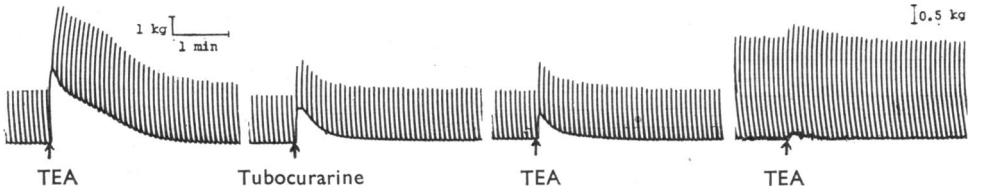

Fig. 11. Effect of TEA on chronically denervated gastrocnemius muscle. The sciatic nerve was cut 11 days. before the experiment. Note the contractile response to tubocurarine (30 μg/kg) in the second tracing and the reduced contractile response to TEA 15 min later. Between third and fourth tracings, two more injections of tubocurarine were given, 100 μg/kg each. All injections of TEA were of 1 mg/kg. Calibrations, 1 min and 1 kg, except for 0.5 kg in last tracing (increased amplification).

response is greater than that of the maximum stimulus-evoked contraction. In four chronically denervated preparations, 1 mg/kg of TEA or 800 μg/kg of PTEA produced contractions of about equal magnitude. However, the contraction produced by 200 μg/kg of 3-OH-PTEA was only about 20% of the contraction produced by a maximum electric stimulus.

In order to determine whether ethonium ions cause potentiation of contraction produced by a maximum stimulus, it was necessary to abolish contraction produced by the drug. For this purpose, repeated doses of tubocurarine were administered until the drug-induced contraction was almost completely blocked. In four preparations, a total dose of 210 to 350 μg/kg of tubocurarine was required to achieve this condition. Note in Fig. 11 that even tubocurarine produces contraction in the chronically denervated muscle. Also note that the contraction produced by 1 mg/kg of TEA is barely detectable after tubocurarine administration, but a minimum potentiation of stimulus-evoked contraction is produced; in four preparations treated with tubocurarine in the manner described, the contractile potentiation produced by 1 mg/kg of TEA ranged from 6 to 11% of control, and the duration of this response was 0.5 to 1.5 min. In two similarly treated denervated preparations, 800 μg/kg of PTEA and 200 μg/kg of 3-OH-PTEA produced potentiation of about the same magnitude and duration as did TEA.

DISCUSSION

A group of ethonium ions and their structural analogues were shown to potentiate neurally-evoked contraction in cat gastrocnemius muscle. The problem raised by the present experiments is whether or not this pharmacologic effect is produced by a single mechanism of action common to all of these quaternary ammonium ions.

Significant differences exist between the slopes of the regression lines relating dose to contractile potentiation and the maximum potentiating responses of these ions. Using these criteria, the entire group of eight compounds are divided into three dose/response classes of potentiating agents: (1) hydroxyanilinium ions with 3-OH-PTEA as prototype; (2) unsubstituted trialkylphenylammonium ions with PTEA as prototype; and (3) tetraalkylammonium ions with TEA as prototype. The hydroxyanilinium ions produce the greatest potentiating effects, and their dose/response regression lines have the steepest slopes; the tetraalkylammonium ions produce minimal potentiating effects and their dose/response regression lines have the flattest slopes. These dose/response results can be interpreted to

suggest that more than one mechanism of potentiating action exists and that each is associated with a characteristic molecular structure.

Differences in regression line slopes and absolute potencies amongst structurally related compounds do not necessarily imply differences in mechanism of action. A flatter slope and lower potency may indicate loss of intrinsic activity (see Ariëns, van Rossum & Simonis, 1957). On this basis, a much greater concentration of TEA-receptor complexes than 3-OH-PTEA-receptor complexes would be required to produce effects of equal magnitude.

The potentiating ions used in these experiments produced repetitive antidromic nerve discharges following a single orthodromic impulse; these antidromic impulses were probably initiated by a prolonged current sink (presynaptic response) located in the motor nerve terminal (Riker *et al.*, 1959a,b; Werner, 1960a,b; Standaert, 1963). The ethonium ions which produce only depression of contraction amplitude do not produce antidromic discharge, which provides additional evidence in support of the hypothesis of a causal relationship between contractile potentiation and the presynaptic response. The order of potency to potentiate contraction is the same as the order of probability of producing antidromic repetitive discharge, namely 3-OH-PDEMA>3-OH-PTEA>PTEA>TEA. In conjunction with paired stimuli, or after tetanic conditioning (Werner, 1960a), the probability that any one ion will produce antidromic repetition increases. Tetanic conditioning also augments the contractile potentiation produced by these compounds (Kuperman & Werner, 1960). After single or repetitive motor nerve stimulation, the excitability of the axon terminals is enhanced (Werner, 1960a,b; Hubbard & Schmidt, 1963; Standaert, 1963), which accounts for the increased intensity of presynaptic drug action under these conditions.

The foregoing evidence suggests that potentiating ethonium ions share a common presynaptic mechanism of action. The quantitative differences amongst these ions with respect to their actions on neurally-evoked contraction and on antidromic repetitive discharge may depend mainly on quantitative differences between their effects on the motor nerve terminals. Inherent in this reasoning is the assumption that the presynaptic response magnitude determines both the magnitude of the contractile potentiation and the probability of occurrence of antidromic repetition.

Although the notion of a common mechanism for ethonium-ion potentiation is appealing and does receive certain experimental support, the striking differences between the effects of ethonium ions after benzoquinonium or tubocurarine injection are not easily accounted for by differences in response magnitude alone. The potentiating effect of 3-OH-PTEA is reduced, abolished or even reversed by benzoquinonium or tubocurarine, whereas the potentiating effect of TEA is relatively unaltered by this treatment. Furthermore, whereas there is a definite ceiling to the antagonism by 3-OH-PTEA of a 70 to 90% paralysis due to tubocurarine or benzoquinonium, there is no ceiling to the reduction of this degree of paralysis by TEA. Similarly, a recent study has shown that, compared to TEA, hydroxyanilinium ions have a limited anticurare action in the frog with either the endplate potential or contraction as the response criterion (Kuperman & Okamoto, 1964).

The suppression of response to 3-OH-PTEA by tubocurarine or benzoquinonium can be explained solely on the basis of an interaction at the motor nerve terminal. Previous experiments have shown that tubocurarine (Riker *et al.*, 1959a; Werner, 1959; Werner & Kuperman, 1963; Standaert, 1964) and benzoquinonium (Blaber & Bowman, 1963)

abolish the antidromic repetitive discharge originating from the axon terminals after application of contractile-potentiating drugs or after tetanic stimulation. The experiments of Blaber & Bowman (1963) indicate that benzoquinonium has a more potent presynaptic effect than has tubocurarine, which accounts for the fact that benzoquinonium suppresses 3-OH-PTEA potentiation in doses which do not depress normal contraction amplitude. It is especially significant that the duration of the presynaptic effects of benzoquinonium or tubocurarine, as signalled by abolition of the antidromic discharge, is about ten- to twenty-times longer than the depression of contraction (Werner & Kuperman, 1963). It is, therefore, understandable that these agents suppress 3-OH-PTEA potentiation for long periods of time after termination of neuromuscular paralysis.

From studies of the interaction between ethonium ions and benzoquinonium or tubocurarine, it seems reasonable to conclude that there are at least two different mechanisms of ethonium-ion action. One mechanism is associated predominantly with 3-OH-PTEA and other hydroxyaniliniums; the other is associated with TEA and other tetraalkylammoniums. Evidence presented in this and previously cited papers strongly implicates the motor nerve terminal as the site of 3-OH-PTEA action. Similar evidence suggests the same site for TEA action and, in fact, a presynaptic effect of TEA at the frog neuromuscular junction has been clearly demonstrated (Koketsu, 1958).

Even if both ions have their site of action in the motor nerve terminal, they could act at different axonal foci and through different mechanisms. Such a scheme could satisfactorily account for the observed pharmacologic differences between the two ethonium ions as follows: (1) the dose/response results show that an effect at the TEA focus results in much weaker potentiation of contraction than an effect at the 3-OH-PTEA focus; (2) the TEA focus is relatively resistant to the action of benzoquinonium or tubocurarine compared to the 3-OH-PTEA focus; (3) an effect at the TEA focus does not result in antidromic discharge whereas an effect at the 3-OH-PTEA focus does—and the antidromic discharge produced by TEA in conjunction with paired stimuli or after tetanic stimulation may be the result of enhancement of a very weak effect at the 3-OH-PTEA focus.

In addition to the evidence for two different presynaptic sites and mechanisms of ethonium ion potentiating action, the present study has revealed certain ethonium ions to be depressants of neuromuscular transmission. Also, the potentiating ions frequently produce depression after administration of benzoquinonium or tubocurarine, and depression is produced by these ions in doses just below those required for potentiation (Kuperman, 1960). Since the unsubstituted trialkylphenylammonium ions have both potentiating and depressing effects, the structure-activity results can be interpreted as follows. The phenolic hydroxyl group and other *meta*-substituents containing an oxygen atom with anionic character (Riker *et al.*, 1957; Kuperman *et al.*, 1961) cause an increase in potentiating activity. This increased potency may be the result of greater affinity between the potentiating ion and a presynaptic receptor. Other *meta*-substituents cause complete loss of potentiating activity and enhancement of depressant potency. There is no consistent pattern with regard to the nature of the " depressing " *meta*-substituents. For example, some are electron repellent and others are electron attracting. Although the mechanism of these depressant effects has not yet been ascertained, the fact that such effects occur reinforces the conclusion that ethonium ions have heterogeneous mechanisms of action at the mammalian neuromuscular junction.

SUMMARY

1. A comparison of certain pharmacologic effects of ethonium ions and their structural analogues was made on a cat nerve-muscle preparation.

2. Some ethonium ions potentiate neurally-evoked contraction, and others depress it. Slopes of the dose/potentiation regression lines and the maximum potentiating effects of triethyl(3-hydroxyphenyl)ammonium (3-OH-PTEA), triethylphenylammonium (PTEA) and tetraethylammonium (TEA) differed from each other significantly.

3. The potentiating response to 3-OH-PTEA was reduced or even reversed after injection of benzoquinonium or tubocurarine, but the relatively weak potentiating response to TEA was unaffected. The reversal of paralysis due to benzoquinonium or tubocurarine by 3-OH-PTEA was relatively limited compared to the antagonism by TEA.

4. The effect of 3-OH-PTEA in producing repetitive antidromic discharges in motor axons was more consistent than the effect of TEA, even in conjunction with paired stimuli.

5. It is concluded that there are at least two mechanisms of ethonium-ion potentiation, one associated with 3-OH-PTEA and the other associated with TEA, and that both occur presynaptically. Although an effect at the 3-OH-PTEA-sensitive focus results in marked contractile potentiation, this site is exquisitely sensitive to the depressant action of benzoquinonium or tubocurarine. On the other hand, an effect at the TEA-sensitive focus causes weak potentiation, but this site is remarkably resistant to the action of stabilizing drugs.

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REFERENCES

ARIËNS, E. J., VAN ROSSUM, J. M. & SIMONIS, A. M. (1957). Affinity, intrinsic activity and drug interactions. *Pharmacol. Rev.*, **9**, 218–236.

BLABER, L. C. & BOWMAN, W. C. (1959). A comparison of the effects of edrophonium and choline in the skeletal muscles of the cat. *Brit. J. Pharmacol.*, **14**, 456–466.

BLABER, L. C. & BOWMAN, W. C. (1963). The effects of some drugs on the repetitive discharges produced in nerve and muscle by anticholinesterases. *Int. J. Neuropharmacol.*, **2**, 1–16.

BOWMAN, W. C. (1958). The neuromuscular blocking action of benzoquinonium chloride in the cat and in the hen. *Brit. J. Pharmacol.*, **13**, 521–530.

HUBBARD, J. I. & SCHMIDT, R. F. (1963). An electrophysiological investigation of mammalian motor nerve terminals. *J. Physiol. (Lond.)*, **166**, 145–167.

KENSLER, C. J. (1950). The anticurare activity of tetraethylammonium ion in the cat. *Brit. J. Pharmacol.*, **5**, 204–209.

KOKETSU, K. (1958). Action of tetraethylammonium chloride on neuromuscular transmission in frogs. *Amer. J. Physiol.*, **193**, 213–218.

KUPERMAN, A. S. (1960). Depression of mammalian neuromuscular transmission. *Fed. Proc.*, **19**, 173.

KUPERMAN, A. S. (1961). Opposite effects on neuromuscular transmission by facilitatory drugs. *Fed. Proc.*, **20**, 304.

KUPERMAN, A. S. (1963). Effects of quaternary ammonium ions on a crustacean nerve-muscle preparation. *J. Pharmacol. exp. Ther.*, **139**, 1–7.

KUPERMAN, A. S., GILL, E. W. & RIKER, W. F. (1961). The relationship between cholinesterase inhibition and drug-induced facilitation of mammalian neuromuscular transmission. *J. Pharmacol. exp. Ther.*, **132**, 65–73.

KUPERMAN, A. S. & OKAMOTO, M. (1964). The relationship between anticurare activity and time course of the endplate potential; a structure-activity approach. *Brit. J. Pharmacol.*, **23**, 575–591.

KUPERMAN, A. S. & WERNER, G. (1960). Interaction between repetitive nerve stimulation and twitch-potentiating agents at the neuromuscular junction. *Nature (Lond.)*, **188**, 1032–1033.

RIKER, W. F., JR., ROBERTS, J., STANDAERT, F. G. & FUJIMORI, H. (1957). The motor nerve terminal as the primary focus for drug-induced facilitation of neuromuscular transmission. *J. Pharmacol. exp. Ther.*, **121**, 286–312.

RIKER, W. F., JR., WERNER, G., ROBERTS, J. & KUPERMAN, A. S. (1959a). The presynaptic element in neuromuscular transmission. *Ann. N.Y. Acad. Sci.*, **81**, 328–344.

RIKER, W. F., JR., WERNER, G., ROBERTS, J. & KUPERMAN, A. S. (1959b). Pharmacologic evidence for the existence of a presynaptic event in neuromuscular transmission. *J. Pharmacol. exp. Ther.*, **125**, 150–158.

STANDAERT, F. G. (1963). Post-tetanic repetitive activity in the cat soleus nerve. *J. gen. Physiol.*, **47**, 53–70.

STANDAERT, F. G. (1964). The action of d-tubocurarine on the motor nerve terminal. *J. Pharmacol. exp. Ther.*, **143**, 181–186.

STOVNER, J. (1958). The anticurare activity of tetraethylammonium (TEA). *Acta pharm. tox.* (*Kbh.*), **14**, 317–332.

WERNER, G. (1959). The presynaptic action of d-tubocurarine and flaxedil at the neuromuscular junction. *Fed. Proc.*, **18**, 458.

WERNER, G. (1960a). Neuromuscular facilitation and antidromic discharges in motor nerves; their relation to activity in motor nerve terminals. *J. Neurophysiol.*, **23**, 171–187.

WERNER, G. (1960b). Generation of antidromic activity in motor nerves. *J. Neurophysiol.*, **23**, 453–461.

WERNER, G. & KUPERMAN, A. S. (1963). Actions at the neuromuscular junction. In *Cholinesterases and Anticholinesterase Agents*, ed. KOELLE, G., suppl. 15, p. 570, Handb. exp. Pharmakol. Heidelberg: Springer-Verlag.