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RELATIONSHIP BETWEEN INHIBITION OF ACETYLCHOLINESTERASE AND RESPONSE OF THE RAT PHRENIC NERVE-DIAPHRAGM PREPARATION TO INDIRECT STIMULATION AT HIGHER FREQUENCIES

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1 Rat isolated diaphragm preparations were stimulated indirectly either intermittently at 20, 50 or 100 Hz or continuously at 0.2 Hz.

2 Addition of 1.8 μM paraoxon (which inhibits acetylcholinesterase by forming a phosphorylated enzyme which undergoes slow spontaneous reactivation) for 5 min to the organ bath produced a failure of the muscle to maintain tetanic tension (tetanic fade, Wedensky inhibition) and potentiated the neuromuscular blocking activity of exogenous acetylcholine. The rates of recovery from both these effects were recorded.

3 In a series of experiments with dyflos (which inhibits acetylcholinesterase by forming a phosphorylated enzyme which does not undergo spontaneous reactivation) the relationship between functional acetylcholinesterase activity and neuromuscular blocking activity of exogenous acetylcholine was also determined.

4 From the data obtained, the relationship between functional acetylcholinesterase activity and tetanic fade was calculated. These calculations show that (i) a considerable reduction in functional acetylcholinesterase activity is required before the diaphragm loses its ability to respond with a sustained tetanus to indirect stimulation at higher frequencies, (ii) the minimum (critical) level of functional acetylcholinesterase activity required for a normal tetanic response is directly related to the frequency of stimulation and (iii) once functional acetylcholinesterase activity has been reduced to the critical level, a very small further reduction leads to a complete tetanic fade.

5 The meaning of functional acetylcholinesterase assays and of conclusions which can be drawn from them, is discussed.

Introduction

Inhibition of acetylcholinesterase (acetylcholine acylhydrolase, E.C. 3.1.1.7) prolongs the postsynaptic action of acetylcholine and this renders striated muscle unable to maintain a contraction in response to nerve stimulation at higher frequencies (tetanic fade; Wedensky inhibition). It also reduces the concentration of exogenous acetylcholine which produces neuromuscular block by postsynaptic depolarization (Burgen & Hobbiger, 1951; Barnes & Duff, 1953). The percentage inhibition of acetylcholinesterase sites in tissues can be determined by measurement of the acetylcholinesterase activity of tissue homogenates (so-called total acetylcholinesterase assay). Studies of the relationship between enzyme inhibition, determined in this way, and tetanic fade (Berry & Evans, 1951; Barnes & Duff, 1953) have shown that only a very small percentage of the acetylcholinesterase sites at the neuromuscular junction is required for a normal muscle response to nerve stimulation at higher frequencies. Subsequently, Koelle & Steiner (1956) and McIsaac & Koelle (1959) provided evidence that only a fraction of all the acetylcholinesterase sites at a cholinergic junction is involved in the hydrolysis of acetylcholine released from the nerve terminals. This fraction they called functional acetylcholinesterase. Incubation of a tissue with intact cholinergic junctions is thought to limit the distribution of substrate (provided it is a quaternary compound) to the enzyme sites which are involved in the hydrolysis of acetylcholine released from nerve terminals and acetylcholinesterase activity recorded under these conditions is referred to as functional acetylcholinesterase activity (Fleischer, Hause, Killos & Harrison, 1960; Barnard & Wieckowski, 1970; Hobbiger & Lancaster, 1971; Mittag, Ehrenpreis & Hehier, 1971).

In this paper results are presented which give information on the correlation between anticholinesterase-induced tetanic fade and functional acetylcholinester-

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ase activity. The studies were carried out on the rat isolated diaphragm preparation, treated with the organophosphate anticholinesterases paraoxon or dyflos. Inhibition of acetylcholinesterase by the former yields a phosphorylated enzyme which slowly undergoes spontaneous reactivation (Burgen & Hobbiger, 1951; see Hobbiger, 1963). Thus the tetanic fade and enhancement of the action of exogenous acetylcholine produced by paraoxon are reversed by removal of the anticholinesterase from the organ bath. On the other hand, dyflos yields a stable phosphorylated enzyme and consequently tetanic fade and enhancement of the action of exogenous acetylcholine are maintained. In the experiments described here, the rat diaphragm preparation was first treated with paraoxon and the rate of recovery from tetanic fade and associated changes in the neuromuscular blocking activity of acetylcholine were determined. The correlation between functional acetylcholinesterase activity and the neuromuscular blocking activity of acetylcholine was then evaluated in preparations treated with dyflos. From these data the relationship between different degrees of tetanic fade and functional acetylcholinesterase activity was calculated.

Methods

Male Wistar rats (200 to 300 g) were used. The left hemidiaphragm with its phrenic nerve and rib cage insertion was suspended in an organ bath containing 40 ml Tyrode solution (mM: NaCl 138.0, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, glucose 11.0). The bath fluid was maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. The resulting pH was 7.15. The phrenic nerve was stimulated through a circular electrode or an open 'Palmer phrenic nerve electrode' connected to an electronic square pulse generator (Palmer) with a constant voltage output. All stimuli were supramaximal (2 to 4 V) and of 0.1 ms duration. For twitch responses the phrenic nerve was stimulated at a frequency of 0.2 Hz. For tetanic responses the rate of stimulation was 20, 50 or 100 Hz, applied for 10 s every 5 min and with only one frequency per diaphragm. Muscle tension was recorded on a smoked drum by a spring-loaded lever giving an 18 fold magnification. Twenty Hz produced an unfused tetanus (clonus), whilst 50 and 100 Hz produced a full (sustained) tetanus. The amplitude of the contraction at the end of the 10 s period of stimulation was used as the measure of the tetanic response.

Acetylcholinesterase assays

The whole diaphragm was perfused *in situ* with Tyrode solution until the effluent was clear. The left hemidiaphragm was then set up for recording of contractions. To obtain enough material for assays of acetylcholinesterase activity, the remainder of the diaphragm was also excised and placed in the same organ bath. As shown by Taugner & Fleckenstein (1950) and Heffron (1972), inhibition of cholinesterases in the isolated diaphragm proceeds at the same rate in the presence and absence of low rates of nerve stimulation.

To determine *functional acetylcholinesterase activity* the rib cage insertion, fatty tissue and phrenic nerve trunk were removed. The muscle was dried with filter paper, weighed and then placed in a vessel containing Tyrode solution (1 ml/100 mg tissue) and acetylcholine in a concentration of 0.05, 0.5 or 5 mM. The vessel was placed in a water bath maintained at 37°C, and the Tyrode solution was gassed continuously with a mixture of 95% O_2 and 5% CO_2. Samples of 0.3 ml were withdrawn from the incubation medium at the start of the incubation and thereafter at intervals, for assay of their acetylcholine content.

To determine *total acetylcholinesterase activity* the muscle was dried with filter paper, weighed and then homogenized in a small volume of Tyrode solution by grinding in a mortar with acid-washed sand. The homogenate was diluted in acetylcholine containing Tyrode solution so as to give 100 mg tissue/2 to 10 ml Tyrode with a concentration of 0.05, 0.5 or 5 mM acetylcholine. Incubation and withdrawal of samples for assay of their acetylcholine content was the same as in experiments with intact diaphragms.

The concentration of acetylcholine in the samples collected from the incubates was determined by bioassay on the frog isolated rectus abdominus muscle preparation. Control experiments showed that assays were unaffected when choline was present in a concentration equal to that of acetylcholine. The same applied to the potassium content of 0.3 ml samples of homogenates. Spontaneous hydrolysis of acetylcholine was negligible. When calculating the hydrolysis of acetylcholine by the intact diaphragm, corrections were made for the amount of acetylcholine withdrawn in individual samples and the half-life of acetylcholine was determined from plots of the acetylcholine content of the incubation medium against the period of incubation. In the case of homogenates the recorded half-life was divided by the volume (in ml) of Tyrode solution used for incubation. This gives the half-life for 100 mg tissue homogenate/ml and as such can be compared directly with the half-life observed in incubations of intact tissue.

Drugs

The following drugs were used: acetylcholine chloride, choline chloride (BDH); tetramono*iso*propyl pyrophosphortetramide (*iso*-OMPA), di*iso*propyl phosphorofluoridate (dyflos) (Koch-Light); diethyl-4-

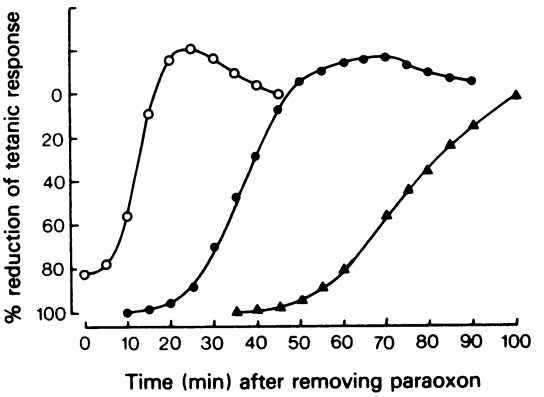

Figure 1 Tetanic responses of the rat diaphragm subsequent to 1.8 μM paraoxon being added to the organ bath and removed after 5 min (zero time). One frequency of stimulation was used per diaphragm and results represent means of two experiments; (○) 20 Hz; (●) 50 Hz; (▲) 100 Hz.

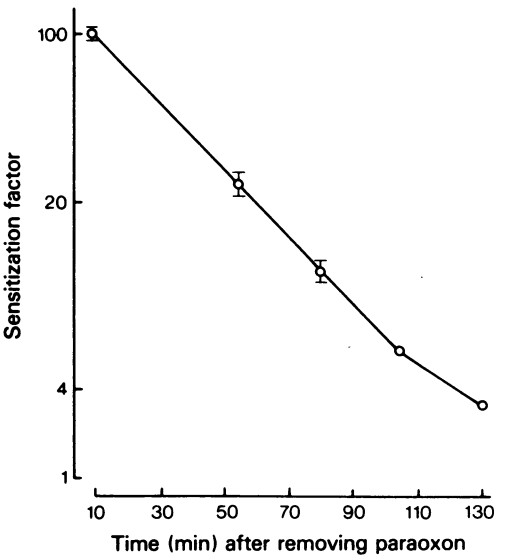

Figure 2 Sensitivity of the rat diaphragm to exogenous acetylcholine subsequent to 1.8 μM paraoxon being added to the organ bath and removed after 5 min. The sensitization factor is the factor by which the neuromuscular blocking activity of exogenous acetylcholine differs from the control value. The bars represent s.e. mean; $n = 15$, except for the final two points, each of which is the mean of two observations (6.5 and 6.1 at 105 and 4.2 and 4.0 at 130 min).

nitrophenylphosphate (paraoxon) (Albright and Wilson). All concentrations given are final concentrations in the organ bath.

Where appropriate, results are expressed as means ± s.e. mean. The statistical significance of differences was determined by Student's t test and a probability of 5% or less was taken as significant.

Results

Effects of paraoxon and dyflos on tetanic responses to indirect stimulation

In control experiments in which the phrenic nerve was stimulated for 10 s every 5 min, at one frequency per diaphragm, the responses to 20, 50 and 100 Hz declined by 15, 5 and 50% respectively over a period of 90 min. At each frequency of stimulation differences between diaphragms in the decline of tetanic tension with time were less than 10%, and the figures represent the means of two experiments at each frequency of stimulation. Corresponding corrections were made in all subsequent studies of the effects of paraoxon and dyflos.

When paraoxon, 1.8 μM, was added to the organ bath and removed after 5 min, the tetanic response to 20 Hz was reduced by 81% 5 min later. Under the same conditions 50 and 100 Hz produced a complete tetanic fade. Subsequently full recovery occurred. As Figure 1 shows, the rate of recovery was inversely related to the rate of stimulation and after complete recovery there was a period during which responses were above control values.

When dyflos, 12 μM, was added to the organ bath and removed after 5 min, no recovery of the tetanic response took place. For example, 5 min after removal of dyflos from the organ bath the tetanic response to 50 Hz was reduced by 57 ± 5%, and at 80 min the reduction amounted to 62 ± 8% ($n = 3$). The P value for this difference is >0.5.

Effects of paraoxon and dyflos upon the neuromuscular blocking action of exogenous acetylcholine and choline

Graded concentrations of acetylcholine were added to the organ bath for 5 min at 10 min intervals and the reductions in twitch tension produced by them were recorded. Paraoxon or dyflos were then added to the organ bath for 5 min. Five min after removal of the organophosphate from the organ bath and at intervals thereafter, the effect of exogenous acetylcholine was tested again, using concentrations which in preliminary experiments produced approximately 50% reduction of twitch tension.

The percentage reductions of twitch tension at the end of each 5 min exposure of the diaphragm to ace-

tylcholine, obtained before addition of paraoxon or dyflos to the organ bath, were plotted against the log of the concentration of acetylcholine used. From these plots the concentration giving 50% reduction of twitch tension (IC_{50}) before treatment of a diaphragm with paraoxon or dyflos was read off and the increase in sensitivity to acetylcholine (called sensitization factor) following such treatment was obtained by interpolation. In the absence of paraoxon or dyflos treatment, the IC_{50} for exogenous acetylcholine increased very slightly with time, confirming previous observations (Heffron, 1972), and sensitization factors for preparations treated with paraoxon or dyflos were corrected for this.

In the absence of acetylcholinesterase inhibition, acetylcholine is rapidly hydrolysed to choline as shown by the IC_{50} values of choline and acetylcholine. In untreated preparations they were 5.5 ± 0.3 mM ($n = 15$) and 4.7 ± 0.5 mM ($n = 5$), respectively with a P value for the difference of >0.5. When paraoxon, 1.8 μM, was added to the organ bath for 5 min, the sensitivity of the diaphragm to exogenous choline was increased by a factor of 3.9 ± 0.5 ($n = 5$) at 10 min after removal of paraoxon from the organ bath and then returned to control levels in 25 min. On the other hand, paraoxon increased the sensitivity to exogenous acetylcholine by a factor of 394 ± 15 ($n = 15$) at 10 min after removing paraoxon from the organ bath. The sensitization factor then declined progressively, falling to 4.2 in 130 min. Correcting values for the transient, non-specific increase in sensitivity as observed with choline, the true sensitization factor at 10 min for exogenous acetylcholine becomes 100. The time course of the decrease in sensitization had first order characteristics, as shown in Figure 2.

Dyflos, added to the organ bath for 5 min in concentrations of up to 18 μM had no effect on the neuromuscular blocking activity of choline. Dyflos, 12 μM, added to the organ bath for 5 min, increased the sensitivity to exogenous acetylcholine by a factor of 14 ± 1.3 ($n = 3$) at 10 min after removal of dyflos from the organ bath. This increase remained unchanged for the next 2 h.

Relationship between sensitization to exogenous acetylcholine and inhibition of acetylcholinesterase

To establish the relationship between sensitization to exogenous acetylcholine and inhibition of acetylcholinesterase, diaphragms were treated for 15 min with 30 μM tetramono*iso*propyl pyrophosphortetramide (*iso*-OMPA). This produces maximal inhibition of cholinesterase (acylcholine acylhydrolase; E.C. 3.1.1.8) without affecting acetylcholinesterase or altering the sensitivity of the diaphragm to exogenous acetylcholine (Heffron, 1972).

In the *iso*-OMPA treated diaphragms the IC_{50} for exogenous acetylcholine was determined first. Subsequently dyflos, 3.6 to 18 μM, was added to the organ bath for 5 min. Ten min after removal of dyflos from the organ bath the IC_{50} of exogenous acetylcholine was redetermined and the sensitization factor was calculated. Finally, the diaphragms were incubated with 0.05 mM acetylcholine, and the rate of hydrolysis was measured.

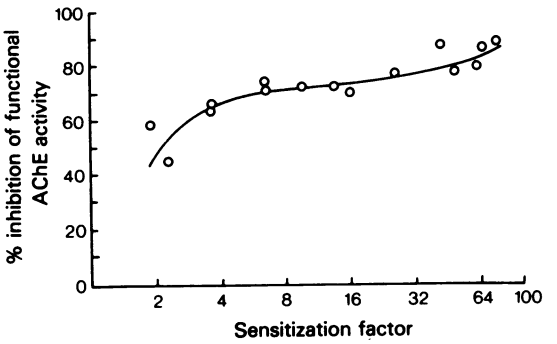

Figure 3 Relationship between the sensitization of the rat diaphragm to exogenous acetylcholine and inhibition of functional acetylcholinesterase (AChE) activity, produced by 3.6 to 18 μM dyflos added to the organ bath for 5 min.

Figure 3 summarizes the results obtained in these studies and shows that marked changes in the sensitization factor were associated with only very small differences in functional acetylcholinesterase activity once the latter was reduced below 60%.

To determine whether studies of the hydrolysis of 0.05 mM acetylcholine gave information which was also applicable to other concentrations of acetylcholine and to establish the relationship between functional and total acetylcholinesterase activity, diaphragms were treated first for 15 min with *iso*-OMPA, 30 μM, and then for 5 min with dyflos, 5 μM. Following this, the rates of hydrolysis of 0.05, 0.5 or 5 mM of acetylcholine by the intact diaphragms and then by homogenates prepared from them were determined. The results obtained in these studies are summarized in Table 1. As the table shows, the reduction by dyflos in the rate of hydrolysis of acetylcholine was independent of the concentration of acetylcholine in each of the two types of assay. However, the ratio (functional acetylcholinesterase activity)/(total acetylcholinesterase activity) was not constant and increased with the concentration of acetylcholine.

Discussion

The diethylphosphoryl acetylcholinesterase formed by paraoxon undergoes spontaneous reactivation. The

rate of reactivation is slow and amounts to less than 5% per hour (see Hobbiger, 1963; 1976; Aldridge & Reiner, 1972). As Figure 1 shows, paraoxon-treated diaphragms recover relatively rapidly from a total inability to maintain a tetanic tension to giving a normal tetanic response, when stimulated indirectly at higher frequencies. The change in acetylcholinesterase activity responsible for the recovery, therefore, must be very small. As Figure 2 shows, paraoxon treatment which causes a complete tetanic fade greatly enhances the potency of exogenous acetylcholine in blocking neuromuscular transmission at low frequencies of stimulation and this sensitization too progressively declines once paraoxon is removed from the organ bath. If we plot percentages of reduction of the tetanic response against the sensitization to exogenous acetylcholine at the times when the responses were recorded, we obtain a result as shown in Figure 4. The relationship between sensitization to exogenous acetylcholine and functional acetylcholinesterase activity was established in dyflos treated diaphragms since the di*iso*propyl phosphoryl acetylcholinesterase formed by dyflos, unlike diethylphosphoryl acetylcholinesterase, does not undergo spontaneous reactivation (see Hobbiger 1963; 1976; Aldridge & Reiner, 1972). This relationship is shown in Figure 3. By substituting in Figure 4 for the sensitization to exogenous acetylcholine the corresponding value of % inhibition of functional acetylcholinesterase activity we obtain Figure 5. This figure shows that (i) a considerable reduction in functional acetylcholinesterase activity is required before the diaphragm loses its ability to respond with a sustained tetanus to indirect stimulation at higher frequencies, (ii) the minimum (critical) level of functional acetylcholinesterase activity required for a normal tetanic response is directly related to the frequency of stimulation and (iii) once functional acetylcholinesterase activity has been reduced to the critical level a very small further reduction leads to a complete tetanic fade.

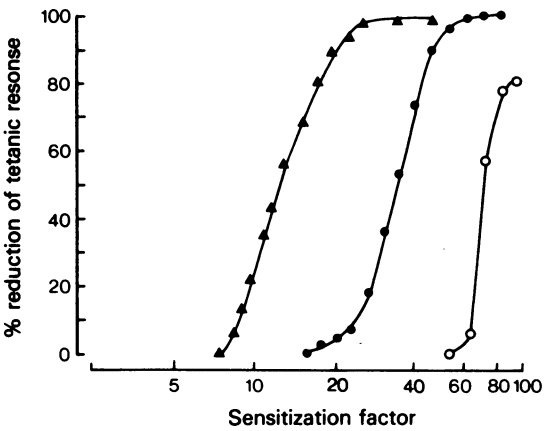

Figure 4 Relationship between sensitization of the rat diaphragm to exogenous acetylcholine and reduction of the tetanic response in diaphragms treated for 5 min with 1.8 μM paraoxon; stimulation at 20 Hz (○); 50 Hz (●); 100 Hz (▲).

The relationship between tetanic fade and acetylcholinesterase activity which we observed, is based on so-called functional acetylcholinesterase assays. In this type of assay intact tissue is incubated with a quaternary substrate, acetylcholine in our case, and the enzyme activity recorded is a function of the rate

Table 1 Effect of dyflos treatment, 5 μM, on the time (in min) required for 50% hydrolysis of acetylcholine, as determined on intact diaphragms (functional acetylcholinesterase (AChE) assay) and homogenates of them (total acetylcholinesterase (AChE) assay)

	Concentration of acetylcholine (mM)	*Half life of acetylcholine (min ± s.e. mean)* *Before dyflos*	*After dyflos for 5 min*	*% Inhibition*
Functional AChE assay (a)	0.05	21.8 ± 1.2	41.0 ± 3.9	47.25
Total AChE assay (b)	0.05	1.3 ± 0.13	5.4 ± 0.9	75.8
Hydrolysis in (a) as % of (b)	0.05	6	13.1	
Functional AChE assay (a)	0.5	32.4 ± 2.5	66.7 ± 9.2	51.2
Total AChE assay (b)	0.5	3.1 ± 0.08	14.7 ± 4.4	79.0
Hydrolysis in (a) as % of (b)	0.5	9.6	22.1	
Functional AChE assay (a)	5	72.0 ± 4.3	139.8 ± 23.4	48.5
Total AChE assay (b)	5	20.6 ± 1.4	78.8 ± 8.1	73.5
Hydrolysis in (a) as % of (b)	5	28.7	56	

$n = 4$ for each concentration of acetylcholine.

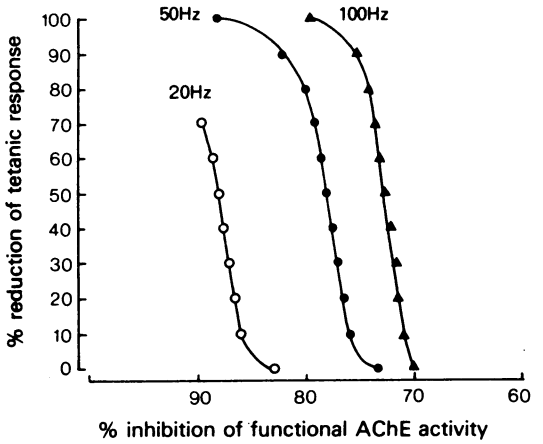

Figure 5 Relationship between inhibition of functional acetylcholinesterase (AChE) activity and reduction of the tetanic responses of the rat diaphragm, stimulated indirectly at 20, 50 or 100 Hz.

and depth of penetration of the substrate into the tissue; the concentration of substrate at individual enzyme sites decreases the further it penetrates and enzyme activity, unlike the activity determined in total acetylcholinesterase assays, is not linearly related to the number of active enzyme sites. Our results, in agreement with observations by Cohen & Posthumus (1957), show that in the absence of acetylcholinesterase inhibition the neuromuscular blocking activities of acetylcholine and choline are comparable. This means that in functional acetylcholinesterase assays, acetylcholine normally only penetrates a very short distance into the endplate. When the enzyme is partly inhibited, the depth of penetration of the substrate is increased and more deeply located enzyme sites also participate in the hydrolysis. At high levels of enzyme inhibition, cellular uptake of acetylcholine will occur and make a contribution to the recorded 'enzyme activity' (Adamič, 1970; Potter, 1970). Conditions in a functional acetylcholinesterase assay, unlike those in a total acetylcholinesterase assay (i.e. the assay using tissue homogenates), therefore, in many respects resemble those applicable to acetylcholine after its release from nerve terminals. If the conditions were identical, the reciprocal value of the fraction to which an anticholinesterase reduces functional acetylcholinesterase activity would be identical with the factor by which the anticholinesterase prolongs cholinoceptor occupancy. Our results show that functional acetylcholinesterase activity can be reduced by approximately 90%. This corresponds to near complete inhibition of the enzyme sites as determined by total acetylcholinesterase assays (Berry & Evans, 1951; Lancaster, 1973). The maximum factor by which cholinoceptor occupancy, as determined by endplate current measurements, can be prolonged by acetylcholinesterase inhibition in the rat diaphragm has yet to be established. Using extracellular recordings, Morrison (1977) obtained a factor of 8 in experiments carried out in the presence of tubocurarine. At the frog neuromuscular junction, using a voltage clamp technique, the factor ranges from 3 to 6 (Katz & Miledi, 1973; Magleby & Terrar, 1975). Assuming that species differences are not responsible for this, the reasons why the relationship between functional acetylcholinesterase activity and prolongation of endplate current after enzyme inhibition deviates from the expected value could be that (i) in the functional acetylcholinesterase assay the substrate (acetylcholine) diffuses into the endplate from the outside whereas the reverse applies to acetylcholine released from nerve terminals, (ii) the distribution of acetylcholinesterase sites relative to cholinoceptor sites at the edge of the endplate might not be the same as at the sites of acetylcholine release from nerve terminals (Matthews-Bellinger & Salpeter, 1978) and (iii) in a functional acetylcholinesterase assay the endplate or part of it is flooded with acetylcholine whereas the experimental evidence supports the view that each of the quanta of acetylcholine released by a nerve impulse initially makes contact with a separate area on the postsynaptic membrane (Hartzell, Kuffler & Yoshikami, 1975; Matthews-Bellinger & Salpeter, 1978).

The view that functional acetylcholinesterase represents a fixed fraction of the total amount of enzyme at the neuromuscular junction (Mittag, Ehrenpreis & Hehier, 1971) is inconsistent with our findings (Table 1) which are in agreement with previous observations by Hobbiger & Lancaster (1971). However, as far as the recruitment of active enzyme sites following partial inhibition of acetylcholinesterase is concerned, it is worth mentioning that there could be differences between the *in vivo* situation and isolated organ preparations, as shown by Lancaster (1973) in studies of the distribution of quaternary anticholinesterases; our findings, therefore, do not necessarily faithfully duplicate the situation *in vivo*.

The results of these studies were briefly reviewed by Hobbiger (1976); in this review the concentration of paraoxon was erroneously given as 0.18 μM.

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