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FUNCTIONAL ACETYLCHOLINESTERASE OF RAT DIAPHRAGM MUSCLE*†

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Abstract—Surface (external) acetylcholinesterase (AChE) and internal AChE of rat diaphragm muscle were assayed separately with radioactive acetyl-β-methylcholine (MeCh) or acetylcholine (ACh) on the intact muscle *in vitro* and on the muscle homogenate. Surface AChE had a K_m for ACh of 1·05 mM and V_{max} of 19·4 μM min^{-1} per diaphragm. External AChE activity increased 27 per cent for a doubling of muscle weight and averaged 20 per cent of total homogenate activity. Phospholine treatment (4×10^{-7} M, 7 min, 30°) potentiated the indirectly elicited twitch response 164 per cent when inhibition of external AChE was 91 per cent and internal AChE 64 per cent, whereas DFP treatment (5×10^{-6} M, 20 min, 30°) potentiated the twitch 257 per cent when inhibition of external AChE was 88 per cent and internal AChE 92 per cent. Inhibition by DFP was reduced by 80 per cent for surface and 32 per cent for internal AChE in the presence of edrophonium (8×10^{-6} M) and by 77 and 0 per cent respectively, in the presence of ACh (2×10^{-3} M). Selective reactivation of DFP-inhibited surface AChE by 2-PAM correlates with recovery from twitch potentiation. The diaphragm muscle gave normal low frequency twitch response if surface AChE inhibition was less than 35 per cent, even when internal AChE was 78–92 per cent inhibited. Surface AChE activity as assayed on intact rat diaphragm muscle *in vitro* with 5×10^{-6} M MeCh as substrate is primarily functional endplate AChE and correlates with cholinergic twitch responses.

IN AN earlier, short communication, we described a sensitive radiometric acetylcholinesterase (AChE) assay,[1] which we have now used to study the surface enzyme in the intact rat diaphragm muscle *in vitro*. The assay method employs radioactive esters of choline as substrates added to the medium bathing the tissue preparation at concentrations in the micromolar range. This method has several advantages over the conventional histochemical and biochemical methods of cholinesterase (ChE) assay: (1) the enzymes remain in their native state bound to membrane sites and are not subject to artifacts introduced by freezing, fixing, homogenization or purification procedures; (2) the assay is a nondestructive method and pharmacological responses to various drugs can be correlated with the drug's effect on the enzymes in the identical tissue; and (3) while the assay of intact tissues *in vitro* does not duplicate the situation *in vivo* it does represent a more physiologic method. Results obtained by this method must be regarded as complementary to the histochemical and conventional biochemical methods for ChE determination where the functionality of the tissue is destroyed. The most serious disadvantage is the problem of slow diffusion of the substrates to enzyme sites within the tissue.

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Striated muscle contains considerable ChE located on the muscle cell membrane and also at intracellular sites. The histochemical methods for AChE show that the external enzyme attached to the membrane surface is located primarily in the mid-region of the cell at the neuromuscular junction, but there is also some present at musculo-tendinous junctions at the end of muscle fibers.[2] This latter site constitutes a significant proportion of the surface enzyme in slow, "red", muscle fibers[3] but is not so significant in the muscle fibers of the rat diaphragm.[4] Thus, the external AChE activity should be related primarily to the number of end plates, but be independent of the muscle weight.

Intracellular AChE of striated muscle fibers has been shown by both histochemical and biochemical methods to be bound to subcellular structures. The enzyme is associated with contractile proteins in the A bands,[5] on myosin,[6] in the sarcoplasmic reticulum,[7] on microsomes, in mitochondria and in the ribonucleoprotein fraction of muscle.[8,9] These sources of enzyme activity constitute the internal pool of AChE. Intact muscle fiber, thus, has both external and internal pools of the enzyme which are separated by a barrier, the muscle membrane. The relative proportions of these 2 enzyme pools and their function at the various sites remain largely unknown.

When radioactive methacholine (MeCh) is used as the substrate at micromolar concentrations, the enzyme assay method does not affect responses of the diaphragm to either electrical stimulation or to applications of drugs. Therefore, the muscle can be set up as a pharmacological preparation concurrently with the enzyme assay. This system can be used to study the relationship of the surface AChE to the functional activity of the neuromuscular junction using a variety of methods.

The object of the present study was to determine the properties and the relative activities of the two enzyme pools and to relate the inhibition of the enzyme pools to a potentiated response of the diaphragm preparation. Each enzyme pool could be studied separately in the following ways. The degree of irreversible inhibition of both pools of AChE for a lipid soluble inhibitor (DFP) was compared to that for a charged quaternary inhibitor of lower lipid solubility (phospholine). Phospholine should selectively inhibit only surface AChE since its penetration of the membrane barrier would occur more slowly and to a lesser degree than that of DFP. Another approach involved the use of excess substrate (ACh) or a poorly penetrating, reversible inhibitor (edrophonium) to protect the external enzyme from DFP inhibition but allowing inhibition of the internal AChE. In a third method, the external enzyme was selectively reactivated by 2-PAM, which penetrates the cell membrane poorly, after inhibition of both the interior and exterior pools of AChE with DFP. In all of these approaches, the response of the diaphragm muscle to phrenic nerve stimulation can be monitored during both the application of the drugs and the enzyme assays.

EXPERIMENTAL

Drugs and substrates. (Acetyl-1-^{14}C)-choline chloride, sp.act. 11 mc/mM, was obtained from Amersham-Searle Corp.; (Acetyl-1-^{14}C)-β-methylcholine iodide (MeCh), sp.act. 4·6 mc/mM, from New England Nuclear Corp. Di-isopropyl fluoro phosphate (DFP) and Pyridine-2-aldoxime methosulfate (2-PAM) were purchased from Aldrich Chemical Co. Phospholine iodide and edrophonium chloride were gifts from Ayerst Laboratories, Inc. and from Hoffman LaRoche, Inc. respectively.

Phrenic-nerve diaphragm preparation. Left hemi-diaphragms of male Sprague–Dawley rats (150–200 g) with attached costal margin and mounted in electrode holders modified after Bülbring[10] were incubated in Tyrode's acetate buffer ($NaC_2H_3O_2 \cdot 3H_2O$, 1·0 g/l.) at 30° and aerated with a mixture of 95% oxygen and 5% carbon dioxide. Surface enzyme assays and drug treatments were carried out on the preparation as described below. The phrenic nerve was continuously stimulated with supramaximal square wave pulses (0·2 msec) at a frequency of 0·2 Hz during the enzyme assays and drug treatments. Contractions were recorded from a baseline tension of 5 g with a Grass FTO3 force-displacement transducer and a Grass Model 7 recorder.

Surface ChE's of diaphragm muscle. The hemi-diaphragm muscles were dissected from the costal margin and phrenic nerve, and suspended from hooks in the assay medium containing radioactive ACh at 10^{-6} M or MeCh at 5×10^{-6} M. A 1-ml aliquot of the bathing medium is added at predetermined time intervals to a short column (15 × 6 mm diameter) of cation exchange resin (Bio-Rex 40, 200–400 mesh) already equilibrated in 0·1 M sodium phosphate buffer, pH 7·4, and the effluent collected in a scintillation vial. The column is then washed successively with 1 ml and 0·5 ml of phosphate buffer and the washings collected in the same vial. Total radioactivity of the bath medium is determined by removing 1 ml from the bath (bypassing the exchange column), and adding it directly to a counting vial containing 1·5 ml of phosphate buffer. Bray's solution is added to each vial (10 ml) and the vials are counted in a liquid scintillation counter at 5°. A plot of radioactivity against time gives a straight line, the slope of which represents tissue hydrolysis rate together with background which was found to be 0·01–0·03% of total substrate per minute for ACh and 0·01–0·003% for MeCh. After completion of the assay (at least four points), the tissue is carefully blotted with absorbent tissue paper and weighed. The calculation of the rate constant (k) is made by the substitution of numerical values in the equation:

$$k = \frac{R \times V_{to} \times 10^{-3}}{W(T - A_{to})}$$

R = rate in counts per minute per milliliter of bath corrected for background autohydrolysis, V_{to} = bath volume in milliliters at zero time, W = tissue weight, T = total counts per minute per milliliter in bath, A_{to} = acetate concentration at zero time in counts per minute per milliliter of bath solution. The rate constant is expressed in units of liters per minute per gram of tissue (wet weight).

Pseudo first-order rate constants for ACh hydrolysis were determined in two groups of rats whose body weights ranged from 150–200 g to 350–450 g and had diaphragm weights averaging approximately 390 and 781 mg respectively.

K_m values were calculated from rates of hydrolysis at four concentrations of ACh in the range 10^{-4} M to 2×10^{-3} M by cumulative addition of nonradioactive substrate to the initial concentration of radioactive substrate; the data were plotted according to the method of Lineweaver and Burk,[11] and K_m and V_{max} were determined graphically.

Comparison of irreversible inhibition of surface and total homogenate AChE. The effect of treatment by DFP (2×10^{-6} M, 15 min) and phospholine iodide (4×10^{-7} M, 7 min) on twitch response and surface AChE of left phrenic nerve hemidiaphragm preparations was determined by assay with MeCh before and after treatment with the irreversible inhibitor. Successive enzyme assays on the same tissue were

separated by a 15-min wash period with three changes of buffer; the same wash procedure was used before and after treatment with DFP or phospholine. The diaphragm muscle was then dissected completely from the costal margin, weighed, and a 0·1% homogenate in buffer was prepared in a motorized Duall homogenizer at 100–200 rev/min for 5 min at 4°. Enzyme activity in one-fifth of the muscle (50 ml of homogenate) was assayed by adding [^{14}C]methacholine to 1 μM concentration. Two-milliliter samples were withdrawn at predetermined time intervals and added to centrifuge tubes containing 0·1 mg of neostigmine to stop hydrolysis, and then centrifuged. Volumes of 1 ml of the supernatant solution were analyzed by separating C_{14} acetic acid from unhydrolyzed substrate on cation exchange columns (Bio-Rex 40 Resin, 200–400 mesh, sodium form) as described above. The right untreated hemi-diaphragm from the same animal was homogenized and assayed exactly as above and used as the control. Inhibition of the internal enzyme was calculated from enzyme activity of the homogenate by correcting for the external AChE activity measured on the identical tissue before homogenization.

Ratio of internal and external AChE activities. Hemi-diaphragm muscles were first assayed for surface AChE with 1 μM C_{14} methacholine or ACh, washed, homogenized and re-assayed for total AChE as described above.

Protection of external AChE by ACh and by edrophonium. After a control assay of surface AChE using methacholine as substrate, the DFP (5×10^{-6} M) was applied in the presence of either ACh (2×10^{-3} M) or edrophonium (8×10^{-6} M) which was added 5 min before the DFP. After the DFP treatment for 20 or 15 min respectively, the drug mixture was thoroughly washed out from the tissue before re-assay of surface AChE. Subsequently, the muscle was homogenized and assayed for internal AChE activity, as described above, using the right untreated hemi-diaphragm as control.

Reactivation of external AChE with 2-PAM. In these experiments, the diaphragm preparation was assayed with methacholine for surface AChE before and after treatment with DFP (5×10^{-6} M, 15 min). 2-PAM (10^{-3} M) was then applied for 10 min and washed out thoroughly before re-assay of the surface AChE activity. Finally, the tissue was homogenized as described above and the AChE activity of the homogenate compared to that of the control right hemi-diaphragm.

RESULTS

Table 1 shows the rate constants for hydrolysis of ACh by diaphragm muscles from 28 animals in two groups of body weight, 150–200 g and 350–450 g. The rate constants

TABLE 1. RELATIONSHIP OF DIAPHRAGM WEIGHT TO ACETYLCHOLINESTERASE ACTIVITY EXPRESSED AS THE PSEUDO FIRST-ORDER RATE CONSTANT k, AND THE K_m AND V_{max} FOR HYDROLYSIS OF ACETYLCHOLINE BY INTACT RAT DIAPHRAGM MUSCLE *in vitro* AT 30°*

Body weight (g)	Diaphragm weight (mg)	k, l., min^{-1} per diaphragm $\times 10^4$	k, l., min^{-1} per g weight $\times 10^4$	V_{max} M, min^{-1} per diaphragm $\times 10^7$	K_m, M, l.$^{-1}$ $\times 10^3$
150–200	390 ± 67 (14)	1·49 ± 0·23	3·89 ± 0·40	1·94 ± 0·3 (6)	1·05 ± 0·3 (6)
350–400	781 ± 98 (14)	1·89 ± 0·37	2·45 ± 0·48		

* Mean ± S.D.

for AChE activity are calculated both on a per gram muscle basis and on a per diaphragm basis for comparison. It is clear that activities are nearly constant on a per diaphragm basis with only a slight increase (27 per cent) for a doubling of the muscle weight. Similarly, the AChE activity of the electroplax is better related to the number of intact cells used rather than to their weight.[12]

In six diaphragm muscles, the K_m and V_{max} for ACh was determined graphically (Table 1). The K_m of 1·05 mM is close to the value for tissue homogenates,[13] but is one-third of the K_m found by Namba and Grob for the synaptic ChE of rat intercostal muscle.[14]

The surface ChE activity in diaphragm was found to be 12 per cent ± 2·5 (*no.* = 5) of the total homogenate activity for ACh as the substrate, and 20 per cent ± 1·5 (*no.* = 14) for methacholine. This would indicate that the synaptic sites on the muscle membranes which are accessible to exogenously applied substrates contain approximately 20 per cent of the total AChE sites in the homogenate.

TABLE 2. EFFECT OF THE VARIOUS DRUG TREATMENTS USED ON TWITCH TENSION AND ON METHACHOLINE HYDROLYSIS BY SURFACE ACETYLCHOLINESTERASE INTACT OF RAT DIAPHRAGM*

Drug treatment	Per cent effect on twitch height	Per cent effect on MeCh hydrolysis
DFP, 5×10^{-6} M, 15 min	+ 271 ± 25 (5)	− 78 ± 2
DFP, 5×10^{-6} M, 20 min	+ 257 ± 21 (5)	− 88 ± 3
Phospholine, 4×10^{-7} M, 7 min	+ 164 ± 28 (9)	− 91 ± 4·5
Edrophonium, 8×10^{-6} M	+ 188 ± 20 (4)	− 78 ± 3
ACh, 2×10^{-3} M, 25 min	No effect (4)	
2-PAM, 1×10^{-3} M, 10 min	No effect (6)	+ 4·9 ± 2·9

* Edrophonium effects were determined in the presence of the drug. Mean ± S.E.

Table 2 lists control experiments showing the effects of the inhibitors and drugs used on indirectly elicited twitch tension and on surface AChE of the rat diaphragm. Twitch heights and enzyme inhibitions were determined on the same preparations. All the inhibitors gave very substantial potentiation of the twitch (164–271 per cent) and also significant inhibition of surface AChE (78–91 per cent). Values for edrophonium are in the presence of the drug, whereas for all other drugs in Table 2, the effect on twitch and AChE was determined after washout. ACh had no direct effect on twitch height apart from a small increase in the first two to four twitches after application. The direct effect of 2-PAM was a depression of twitch height of about 10 per cent.

We attempted to use phospholine as a selective inhibitor of external AChE to correlate the external enzyme activity with the twitch response of the diaphragm. Table 3 shows the degree of inhibition found for the external and internal AChE after application of phospholine iodide. This treatment with the inhibitor (4×10^{-7} M, 7 min, 30°) potentiates the indirectly elicited twitch by about 164 per cent (Table 2). A considerable degree of inhibition of internal enzyme by phospholine was found. The difference in inhibition between the pools of enzyme averaged 27 per cent when using either methacholine or ACh as the substrate showing considerable penetration of phospholine into muscle cells.

TABLE 3. PERCENTAGE INHIBITION OF TOTAL (HOMOGENATE), EXTRACELLULAR (EXTERNAL) AND INTRACELLULAR (INTERNAL) ACETYLCHOLINESTERASE IN RAT DIAPHRAGM BY PHOSPHOLINE TREATMENT (4×10^{-7} M, 7 min, 30°)

Substrate	Total AChE (Homogenate)	External AChE (Surface)	Internal AChE (Calculated)	Ext.–int. (Difference)
MeCh	81	97	76	21
MeCh	70	90	60	30
MeCh	60	85	52	33
MeCh	70	89	59	30
MeCh	81	93	66	27
ACh	68	91	64	27
ACh	60	88	56	32
ACh	68	93	64	29
ACh	69	85	67	18

There is a small degree of selectivity for the external AChE by comparison to DFP treatment (5×10^{-6} M, 20 min) which inhibited surface AChE 88 per cent $\pm$ 3 and internal AChE 92 per cent $\pm$ 3 (*no.* = 4).

Edrophonium, a reversible quaternary AChE inhibitor, can be used to protect the external AChE from DFP inhibition. Results from these experiments are given in Table 4. In the absence of edrophonium, DFP (5×10^{-6} M, 15 min, 30°) gave 78 per cent inhibition of both internal and external AChE with concomitant maximum potentiation of the indirect twitch of 270 per cent. Edrophonium (8×10^{-6} M) by itself potentiated the twitch by 188 per cent (Table 2). The presence of edrophonium reduces the irreversible external AChE inhibition by DFP very significantly from 78 to about 15 per cent. There is also some degree of protection of the internal enzyme, DFP inhibition being reduced from 78 to about 53 per cent. After washout of the mixture of edrophonium and DFP, no potentiation of the twitch was observed.

Table 5 shows results of similar experiments in which a high concentration of ACh was used as the protecting agent in the presence of DFP. After washing out the

TABLE 4. EDROPHONIUM (EDR 8×10^{-6} M) PROTECTION OF EXTRACELLULAR (EXTERNAL) ACETYLCHOLENESTERASE OF RAT DIAPHRAGM AGAINST IRREVERSIBLE INHIBITION BY DFP (5×10^{-6} M, 15 min, 30°)

Total AChE (Homogenate)	External AChE (Surface)		Internal AChE (Calculated)	
EDR + DFP	DFP	EDR + DFP	DFP	EDR + DFP
43*		19		50
50		19		63
	78 ± 2†		78 ± 3	
		14		
38		9		46

* Numerical values are percentage inhibition of MeCh hydrolysis.
† Mean value from Table 2.

TABLE 5. ACETYLCHOLINE (2×10^{-3} M) PROTECTION OF EXTRACELLULAR (EXTERNAL) ACETYLCHOLINESTERASE OF RAT DIAPHRAGM AGAINST IRREVERSIBLE INHIBITION BY DFP (5×10^{-6} M, 20 min, 30°)

Total AChE (Homogenate)	External AChE (Surface)		Internal AChE (Calculated)	
ACh + DFP	DFP	ACh + DFP	DFP	ACh + DFP
81*		9		97
83		22		95
	88 ± 3†		92 ± 3	
67		31		86
77		20		93

* Numerical values are percentage inhibition of MeCh hydrolysis.
† Mean value from Table 2.

ACh and DFP mixture, re-assay showed that the external enzyme was very significantly protected against DFP inhibition, while there was no protection of the internal enzyme. After washout of the ACh and DFP mixture, no irreversible potentiation of the twitch response was observed.

Experiments with reactivation of inhibited external AChE by 2-PAM confirmed the results of the protection experiments. Hemi-diaphragms were inhibited with DFP to the point of maximum twitch potentiation, washed thoroughly and inhibition of surface AChE determined. The preparations were then treated with 2-PAM (10^{-3} M) until the augmented twitch had recovered (10 min), washed free from 2-PAM and the surface activity re-assayed. The tissue was then homogenized and the enzyme activity compared to that of an homogenate of the untreated control hemi-diaphragm. The twitch responses are shown in the lower panel of Fig. 1 and enzyme activities in the

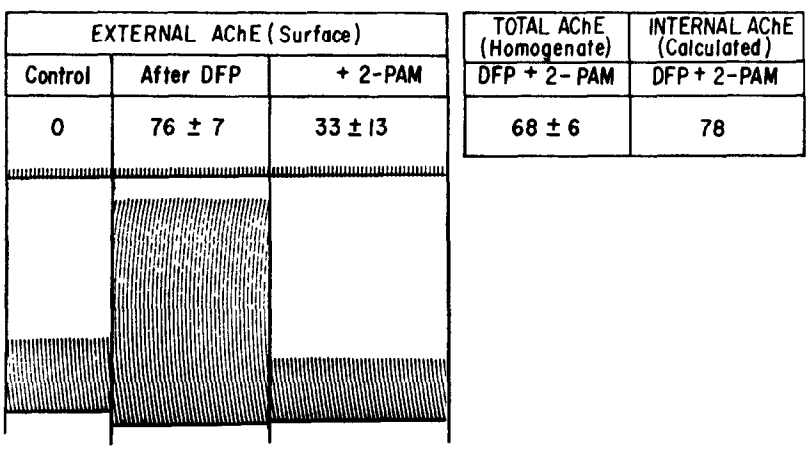

FIG. 1. Effect of 2-PAM (10^{-3} M, 10 min) on per cent inhibition of surface and internal acetylcholinesterase by DFP (5×10^{-6} M, 15 min); correlation with effect on twitch height. Mean and S.D. from five experiments.

upper panel. The results show that recovery from twitch potentiation correlates with 57 per cent reactivation of external inhibited AChE, and the internal enzyme pool remains irreversibly inhibited, showing no reactivation.

DISCUSSION

The object in utilizing this assay method on intact tissues is to study the properties of ChE's at extracellular locations only, primarily those at end-plates. The major barriers to penetration of exogenously applied substrates into tissues are the cell membranes. The substrates used are positively charged ions and penetrate into cells poorly.[12] The loss of ACh because of penetration into intact muscle cells under the assay conditions was found to be very small in comparison to the amount hydrolyzed by membrane-bound AChE.[1] Thus, the intracellular pool of ChE is inaccessible to the substrate and would not be assayed by this method.

The most serious problem with the assay procedure is that the rate of hydrolysis may be limited by diffusion of substrate into the extracellular space and into the synaptic clefts. This limits the method to thin sheets of muscle but, even in the diaphragm which is 10–20 fibers thick, diffusion may be an important factor.

The role of diffusion has been evaluated by a study of the temperature dependence, K_m and V_{max} for ACh hydrolysis by intact diaphragm. The temperature effect on rates of hydrolysis between 15° and 35° gave straight line slopes for the Arrhenius plots for substrate concentrations below 5×10^{-4} M. The slope of the plot gave an activation energy of 8 kcal/mole for ACh hydrolysis. This indicates that the rate-limiting step is a chemical reaction, but diffusion factors may partly determine the rate of hydrolysis. Recently, the diffusivity of ions and also nonelectrolytes has been determined inside muscle cells.[15] Diffusion rates were decreased from that in water by only a factor of two and, thus, it is possible that diffusivities in the extracellular space may be reduced by a similar, small extent. The problem of substrate diffusion is analogous to the limited access problem when different drugs are applied to an intact pharmacological preparation *in vitro*. The absolute concentration of drugs at their cellular site of action cannot be determined and only relative activities are obtained. Likewise, the ChE assay in intact tissues gives relative enzyme activities. When using low substrate concentrations, relative enzyme activities are conveniently expressed in terms of pseudo first-order rate constants, which are independent of substrate concentration below 10^{-4} M.

From the V_{max} and K_m data, an estimate can be made of the absolute ChE activities. From the number of end-plates in rat diaphragm muscle[16] and the V_{max}, the number of ACh molecules hydrolyzed by a single end-plate in 1 msec is $9{\cdot}7 \times 10^7$. This compares with literature values of $2{\cdot}9 \times 10^7$ in mouse diaphragm and $6{\cdot}0 \times 10^7$ in mouse gastrocnemius,[17] $3{\cdot}5 \times 10^7$ in rat rectus abdominus muscle,[18] $1{\cdot}8 \times 10^7$ in rat diaphragm muscle[19] and $27{\cdot}0 \times 10^7$ in rat intercostal muscle.[14]

Based on assays of surface AChE, the external membrane surface contains about 20 per cent of the total AChE sites in the muscle homogenates. However, this value would be an underestimate if limited diffusion influenced the penetration of substrate to sites in deeper areas of muscle fibers. The results for temperature dependence of hydrolysis and the number of AChE sites discussed above indicate that diffusion may be a minimal factor. In addition, this result is in agreement with the experiments of Namba and Grob,[14] who found that the end-plate membrane fraction from rat

intercostal muscle contained 20 per cent of the AChE activity of the total muscle homogenate. Barstad compared the degree of DFP inhibition of homogenates of diaphragm muscle after the DFP was applied to the intact muscle, both by itself and also in the presence of ACh (2×10^{-3} M).[20] The high ACh concentration protects end-plate AChE from inhibition by DFP.[21] The degrees of inhibition found by Barstad differed by 16 per cent, which represents the fraction of the total AChE located at end-plates and on the muscle membrane. These results show that the AChE activity measured by the assay of surface enzyme is of the order expected for the end-plate AChE of the muscle.

In order to correlate this AChE pool with the functional activity of end-plates, methods similar to those employed by Koelle and Koelle were used.[22] McIsaac and Koelle studied the effect of lipid-penetrating inhibitor and also that of a poorly penetrating inhibitor (phospholine) on ganglion cells by the histochemical method.[23] They were able to show that, in the ciliary and in the superior cervical ganglion of the cat, inhibition of the intracellular AChE had no effect on ganglionic transmission, in contrast to the inhibition of extracellular AChE. The external fraction of the enzyme was, thus, termed "functional" and the internal AChE "nonfunctional" or "reserve".

The highly lipid soluble inhibitor, DFP, gave a similar degree of inhibition for both internal and external pools of AChE (Table 4). Under these experimental conditions, the concentration of radioactive DFP within the muscle was 6-fold greater than that of the bathing solution after a 20-min exposure. DFP taken up into tissue lipids may be liberated on subsequent homogenization and cause a further inhibition of AChE. Additional enzyme inhibition was not found in these experiments, suggesting that most of the DFP remains dissolved in lipid, despite homogenization. Even if DFP entered into the aqueous phase, the concentration would be very low (0·1% weight/vol. homogenates were made) and the rate of AChE inhibition would be extremely slow.

On the diaphragm, phospholine was not a sufficiently selective inhibitor of the extracellular pool of AChE. When inhibition of external AChE was 91 per cent, the internal AChE was inhibited by 64 per cent, and the potentiated twitch could not be unequivocally correlated to inhibition of either enzyme pool. Edrophonium protection of the external AChE from irreversible DFP inhibition was more selective. Inhibition was reduced by 80 per cent for surface enzyme and by 32 per cent for internal AChE in the presence of edrophonium (Table 4). After washout of the drug mixture, the twitch response of the muscle returned to the control tension, compared to a 271 per cent potentiation by DFP alone. These experiments indicate that protection of the external enzyme against irreversible inhibition also protects against irreversible twitch potentiation by DFP. Edrophonium is also able to penetrate into the muscle fiber to some extent during the 20-min application of the drug at 8×10^{-6} M and to partially protect internal AChE.

High concentrations of ACh have been shown by histochemical methods to protect end-plate AChE against DFP inhibition.[21] Protection experiments with 2 mM ACh gave a reduction of inhibition by DFP of 77 per cent for the external AChE and no protection of the internal enzyme pool (Table 5). The twitch response was not potentiated after washout of the drug mixture, whereas DFP treatment alone potentiated the twitch 257 per cent. Both types of protection experiments show clearly that there is no effect on transmission or on twitch response at a time when internal AChE of the

diaphragm was inhibited up to 90 per cent. The small extent of inhibition of surface AChE (10–20 per cent) had no effect on response, since single twitch responses only become potentiated when more than 35 per cent of the external AChE is inhibited (Fig. 1).[24]

A selective reactivation of inhibited external AChE by 2-PAM has been found in brain slices[25] and also occurs in the diaphragm (Fig. 1). A 57 per cent reactivation of the external DFP-inhibited AChE is accompanied by a complete reversal of the potentiated twitch, while the internal AChE shows no reactivation. Barnard and Wieckowski[26] have recently shown, by autoradiographic methods, that the effect of DFP on impulse transmission and its reversal by 2-PAM correlate with the binding and removal, respectively, of DFP groups at the end-plate. Threshold for potentiation of low frequency twitch response occurred when 65 per cent of the DFP-reactive sites were occupied, whereas the present results indicate that threshold occurs when 30–40 per cent of the enzymatic activity toward MeCh is inhibited. The difference between results of the two methods may indicate the presence of AChE isoenzymes with different turnover numbers within end-plates.

A comparison of the internal and external enzyme pools demonstrates that charged quaternary nitrogen drugs are able to penetrate into the interior of muscle cells depending on the lipophilic nature of the molecules, e.g. phospholine, a diethyl phosphoryl ester of thiocholine, penetrates to a greater extent than edrophonium, which has a phenolic group, or ACh, which penetrates least.

These experimental results show that the surface AChE measured by this assay method appears to be primarily the functional synaptic enzyme located at the end-plate and correlates with the cholinergic responses of the muscle. Conventional AChE assay procedures, which are either spectrophotometric or depend on a change in hydrogen ion or CO_2 production, cannot be used on respiring tissues at very low substrate concentrations. Despite several inherent difficulties, this new procedure provides data on the functional activity and the tissue, and is useful as a means of relating the extent of functional enzyme reaction to the pharmacological activity of drugs.

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