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Study of Frog Sartorius Muscle Acetylcholine Receptor Using the Irreversible Inhibitor TDF*

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Summary. The response of normal and denervated frog sartorius muscles to several agonists differing in intrinsic activity was studied using the fluid electrode technique. The response to carbamylcholine could be irreversibly blocked by exposure of the muscles to *p*-trimethylammoniumbenzenediazonium difluoroborate (TDF), but the response could be protected from blockage by agonists and antagonists indicating that both TDF and these ligands act at the acetylcholine binding site of the receptor. It is shown that specific reversible binding of the trimethylammonium group of TDF to the receptor plays little or no role in the irreversible reaction of TDF with the receptor, which accounts for the extremely low specificity of its reaction with the receptor.

Affinity labeling methods are potentially very useful for the specific identification and isolation of receptor proteins in membranes. An affinity labeling reagent for a receptor is a chemically reactive derivative of a specific ligand which normally binds to that receptor. Specific reversible binding of the labeling reagent to the active site of the receptor increases the concentration within the site of the reactive functional group on the labeling reagent, thereby enhancing the rate of its reaction with some amino acid residues in the site over the rates with groups outside the site. So, designation of an irreversible receptor inhibitor as an affinity labeling reagent requires that it be shown not only (1) that the reagent reacts in the ligand binding site of the receptor, but also (2) that the rate of its reaction depends on specific reversible binding. If the reactive functional group of an affinity labeling reagent is oriented so that it can react efficiently, the specificity of its reaction is proportional to the affinity with which it is reversibly bound

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[13]. Attaining a large degree of specificity is the most serious problem besetting the application of affinity labeling methods to the labeling of receptor proteins in intact membranes or membrane fragments [14]. Because of the great preponderance of nonspecific proteins and other reactive compounds compared to the expected amounts of most receptor proteins in a membrane, the specific affinity labeling reaction may not be detectable against the background of all the nonspecific reactions which the affinity labeling reagent may undergo.

A variety of reagents have been reported to behave as affinity labeling reagents of AChR[1] activity [3, 6]. Some time ago [15] it was suggested that TDF, which had been successfully used as an affinity labeling reagent for specific antibodies [5], might be a useful reagent to label AChR. The effects of this reagent on the AChR of eel electroplax have been studied [3]. In this paper, we describe some properties of the AChR of frog sartorius neuromuscular junction and the effects of TDF on its activity. Further studies of this AChR using other reagents are described in a paper to follow [9] and in Kiefer, Lindstrom, Lennox and Singer [7].

Materials and Methods

The AChR activity of frog sartorius muscle was studied by a modification of the fluid electrode technique of Fatt [4]. Four muscles were studied simultaneously, each on an apparatus as illustrated in Fig. 1. A muscle was firmly suspended in the plexiglass cylinder by threads tied through the pelvic cartilage at the upper end and around the

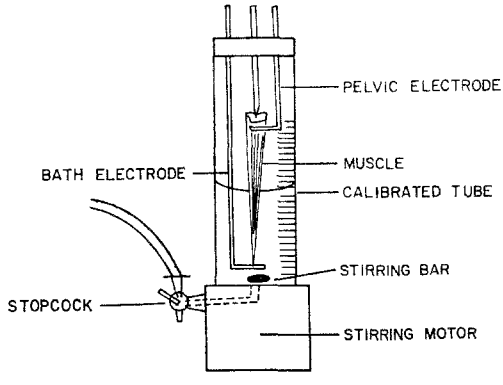


Fig. 1. Fluid electrode apparatus. The calibrated plexiglass cylinder has a volume of about 10 ml. The battery-operated stirring motor is run constantly during the experiment

1 Abbreviations used are: AChR, acetylcholine receptor; ACh, acetylcholine; TDF, *p*-trimethylammoniumbenzenediazonium difluoroborate; MNBDF, *m*-nitrobenzenediazonium fluoroborate; PNBDF, *p*-nitrobenzenediazonium fluoroborate; Carb, carbamylcholine.

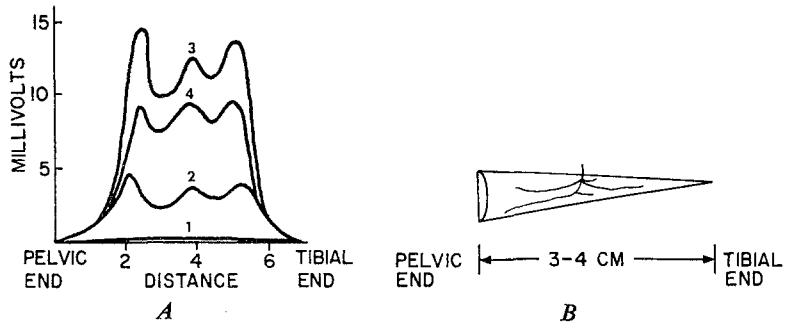


Fig. 2. Distribution of AChR activity on a sartorius muscle. (*A*) The pattern of depolarization along the muscle is shown as the solution is drained at a constant rate and the meniscus falls from the pelvic to the tibial end. The abscissa is a measure of distance along the muscle but is recorded in seconds as the meniscus falls at an approximately constant rate. Potentials were measured under the following conditions: (1) Ringer's solution alone, (2) after 3-min immersion in Ringer's solution containing Carb at 2.5×10^{-5}, (3) after 9 min of Carb, (4) after 12 min of Carb, showing desensitization. (*B*) Branching of large myelinated segments of nerve on the undersurface of the sartorius muscle which gives rise to the multiple sites of innervation along the fibers

tendon at the lower (tibial) end. Glass tubes filled with 4% agar in Ringer's solution and containing coils of chlorided silver wire served as electrodes. The pelvic electrode, which contacts the muscle through a silk thread stiffened with agar, was placed on an end plate-free region of the muscle and the potential difference between the muscle membrane at the level of the pelvic electrode and the muscle membrane at the level of the meniscus of the Ringer's solution was measured. If the muscle is bathed in a Ringer's solution of agonist, a depolarization of the muscle fibers develops in the areas where end plates are concentrated, which results in a potential difference between the fiber membrane at the pelvic electrode which has a normal resting potential, and depolarized membrane at the end plates. The meniscus of the Ringer's could be maintained at any point along the muscle or moved continuously along the surface by regulating the influx or efflux of Ringer's solution through the valve in the bottom of the cylinder. The potential difference was recorded through a 500 MΩ input impedance operational amplifier (with an amplification of unity) which was connected to a Honeywell 530 TM X-Y plotter.

The response to agonists was measured by two methods. In one, the distribution of the potential difference as a function of position along the length of the muscle was measured. Draining a Ringer's solution of agonist bathing the muscle moved the solution meniscus down the muscle allowing potential measurements along the length of the muscle as illustrated in Fig. 2. In the second method, the response at a fixed point on the muscle was measured as a function of time by maintaining the agonist solution at a constant level on the muscle as shown in Fig. 3. The fixed point method was used for most measurements reported in this paper. The point on the muscle at which the response was measured was the tibial-most concentration of end plates, which was located on each muscle by using the moving meniscus technique.

Chronic denervation of sartorius muscles was performed by excising a centimeter of the sciatic nerve at the point where it enters the sartorius muscle and also excising a centimeter from the main nerve trunk to that leg at the level of the pelvis. The contralateral leg was left unoperated and used as the normal control.

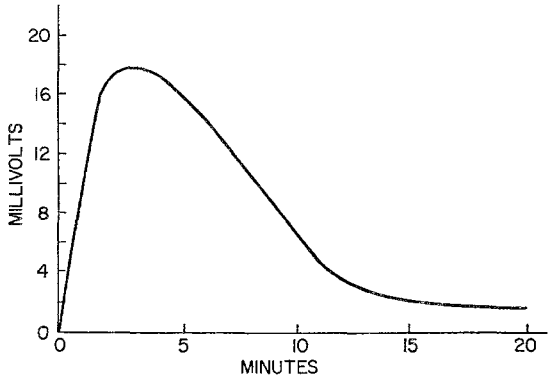


Fig. 3. ACh response at the tibial peak of sartorius muscle AChR activity. ACh at 5×10^{-5} M is applied in Ringer's solution containing neostigmine at 5×10^{-5} M to inhibit ACh esterase

Frog Ringer's solution consisted of 115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl and 10 mM dextrose buffered with 3 mM Na phosphate, pH 7.0. All reagents were applied to muscles in Ringer's solution lacking dextrose. A "30 mM K^+ Ringer's" was identical to that above, except that the concentrations of KCl and NaCl were 30 mM and 87.5 mM, respectively.

TDF was synthesized by Traylor and Singer [15] and their method was used for the synthesis of MNBDF and PNBDF.

Results

Normal Response to Agonists

The fluid electrode technique [4] was used in these experiments because it offered a convenient method for assaying the AChR activity of a well-characterized pure twitch fiber muscle like the frog sartorius. The response measured is the result of AChR-induced depolarization, so it is unaffected by agents which block conduction or alter the contractile mechanisms of the muscle. This technique, however, has the disadvantage as compared with microelectrode techniques that the response measured is the summed response of many fibers depolarizing and desensitizing at different rates and the absolute value of the measured potential difference can not be quantitatively compared with the resting potential.

Bathing a sartorius muscle in a Ringer's solution of agonist causes the muscle fibers to depolarize in areas where end plates are concentrated. The pattern of depolarization along the muscle which develops is illustrated in Fig. 2. The pattern is usually similar for contralateral muscles from a single frog. Thin muscles from small frogs give the largest potentials and most

clearly defined patterns. If the muscles are bathed in Ringer's solution after exposure to agonist, the potential difference gradually returns to its original value near zero. Restoration of the original resting potential after exposure to low concentrations of agonist may require less than 5 min, but after high concentrations more than 30 min may be required.

Other aspects of the response of sartorius muscle to agonists can be illustrated by introducing a Ringer's solution of ACh with the meniscus at the level of the tibial-most concentration of end plates and recording the depolarization of the fibers at that point as a function of time (Fig. 3). Neostigmine inhibition of ACh esterase activity is required to observe a response to readily hydrolyzed agonists like ACh. Because the observed potential difference results from the summed responses of many muscle fibers, the time course of the response or its magnitude do not reflect the changes observed at a single end plate. Note that the response rapidly decreases despite the continued presence of agonist. Desensitization is thought to result from the formation of transiently inactive AChR's after the binding of agonists [12]. The rate of desensitization is highest for the highest affinity agonists like ACh, and increases with the concentration of agonist. Tetrodotoxin at 4×10^{-7} M completely blocks electrical excitability of the muscle, but does not affect the response to agonists. The response to a particular concentration of agonist is defined as the maximum potential difference attained in the presence of a particular concentration of agonist, corrected for any potential difference present at that level before agonist is added. The initial potential difference after prolonged rinsing in Ringer's solution is usually less than 1 mV. There is substantial variance in the potential difference elicited by a particular concentration of Carb depending mostly on the size of the muscles from the particular batch of frogs used. In general, thinner muscles give larger potential differences. The absolute value of the response to 2.5×10^{-3} M Carb averaged over many batches of frogs is 20.4 ± 4.7 mV ($n = 421$)[2]. The standard error in this case is 23%, but the response of the two muscles from a single frog differed on the average by $9.4 \pm 9.6\%$ ($n = 181$), and 37% of these pairs differed by less than 5%. To reduce the error further, the response of a muscle to an agonist was always expressed as a per cent of the response of that muscle to 2.5×10^{-3} M Carb. Also, when detailed comparisons between two treatments were desired, they were performed on paired muscles from a single frog. The best preparations could be used for up to 12 hr, although they rarely were studied for periods longer than 6 hr. The reproducibility of the response to 2×10^{-6} M ACh in the presence of 10^{-5} M neostigmine was examined on

2 Value $\pm$ standard error; (n = number of experiments).

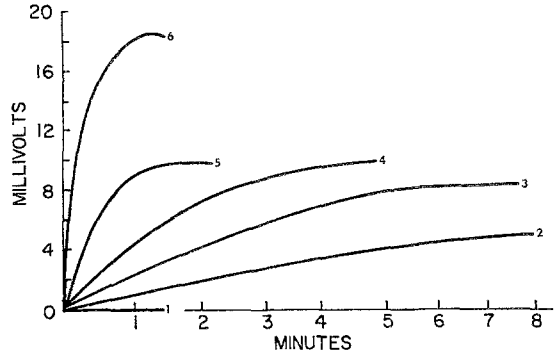


Fig. 4. Nicotine and Carb responses at the tibial peak of AChR activity of the sartorius muscle. The response to several concentrations of nicotine was measured: (1) 2×10^{-6} M, (2) 1.6×10^{-5} M, (3) 2.5×10^{-5} M, (4) 1×10^{-4} M, (5) 2×10^{-4} M. Then the maximum response to Carb was measured: (6) 2.5×10^{-3} M

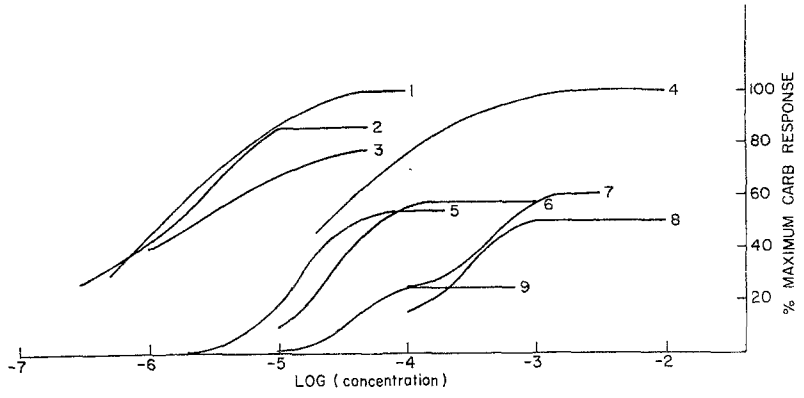


Fig. 5. Dose-response curves for several agonists of the sartorius muscle AChR. Each curve shows the responses at the tibial peak of AChR activity from one muscle relative to the response obtained with 2.5×10^{-3} M Carb on that muscle. (1) ACh, (2) propionylcholine, (3) butyrylcholine, (4) Carb, (5) nicotine, (6) dimethylphenylpiperazinium, (7) acetyl-β-methylcholine, (8) phenyltrimethylammonium, (9) decamethonium

one muscle. During the first 4 hr, the response was $100 \pm 1.8\%$ (n = 4) of the initial value, and at 8 and 9.5 hr it was 105% of the initial response.

Not all agonists produce as large a maximum response as ACh and Carb (Figs. 4 and 5). For example, nicotine is a partial agonist with about half of the intrinsic activity [2] of Carb or ACh. Here, intrinsic activity of a ligand is defined as the ratio of the maximum response to a ligand to the maximum response to Carb so that an antagonist has an intrinsic activity of 0. Fig. 5 shows dose-response curves for several agonists and partial agonists. All the "nicotinic" agonists (decamethonium, nicotine, dimethyl-

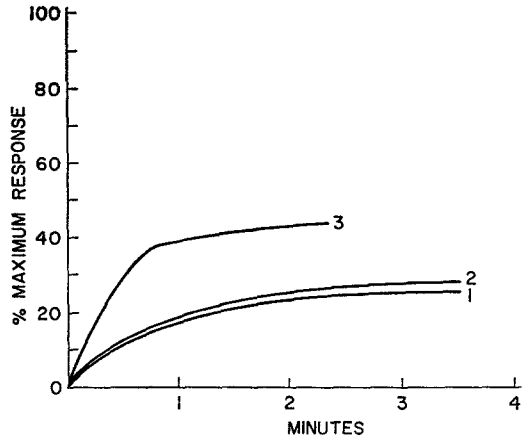


Fig. 6. Inhibition of the response to ACh by phenyltrimethylammonium. A near maximum response to phenyltrimethylammonium given by this partial agonist at 10^{-3} M is shown in (1). (2) shows the response to 10^{-3} M phenyltrimethylammonium and 2×10^{-6} M ACh in the presence of 10^{-4} M neostigmine. That concentration of ACh in the absence of phenyltrimethylammonium gives about 50% of the maximum Carb response. (3) shows the response to 10^{-3} M phenyltrimethylammonium and 10^{-5} M ACh. That concentration of ACh alone gives about 88% of the maximum Carb response

phenylpiperazinium, phenyltrimethylammonium) and the "muscarinic" agonist acetyl-β-methylcholine are partial agonists. This is unlikely to result from the presence of both "nicotinic" and "muscarinic" AChR which separately can only give partial responses because systematic changes in the structure of a ligand can progressively alter the intrinsic activity of a ligand by small increments. The intrinsic activity of the choline esters gradually decreases from acetyl- to propyl- to butyryl-choline. Nonanoylcholine has an intrinsic activity of less than 0.1 and desensitizes very rapidly. Decanoylcholine has an intrinsic activity of 0. This suggests a continuous transition from agonist to antagonist with a continuous elongation of the alkyl chain as a result of altered intrinsic activity at a single type of AChR. That agonists and partial agonists act at the same site is also suggested by the experiment shown in Fig. 6. The partial agonist phenyltrimethylammonium can completely inhibit the response to low concentrations of ACh, but higher concentrations of ACh can compete for AChR sites giving a larger response than the partial agonist alone.

Ligands differ greatly not only in intrinsic activity, but also in affinity for the AChR. If it is assumed that partial and complete agonists bind to the same AChR, and that the concentration producing a half maximal response for that partial or complete agonist equals the apparent affinity

constant, the results shown in Fig. 5 or listed in column 3 of Table 1 illustrate the wide range of affinity of the AChR for various ligands. The apparent affinity of a ligand is fairly constant between several muscles. For example, for Carb $K_D = 2.7 \pm 1.3 \times 10^{-5}$ M (n = 10).

Responses of Denervated Muscles to Agonists

Chronically denervated muscles become hypersensitive to agonists due to formation of additional receptors outside the end plate region [11]. Muscles which have been denervated 6 to 12 weeks respond to concentrations of Carb 10-fold or more lower than normal and give larger maximum responses. However, these denervated muscles still give a pattern of peaks when the distribution of agonist-induced depolarization along the length of the muscles is measured, indicating that although the AChR's may have spread somewhat from the end plate region, the surface of the muscle does not become uniformly sensitive (Figs. 7 and 8). However, the potential caused by 30 mM K^+ Ringer's is the same in normal and contralateral denervated muscles.

The intrinsic activity of the partial agonists decamethonium and dimethylphenylpiperazinium increases after denervation. Normal muscles have an affinity constant for dimethylphenylpiperazinium of $K_D = 3.5 \pm 0.96 \times 10^{-5}$ (n = 3) and have an intrinsic activity of 0.63 ± 0.05 (n = 3), whereas the contralateral muscles denervated 11 to 13 weeks have $K_D = 4.5 \pm 1.1 \times 10^{-6}$ M

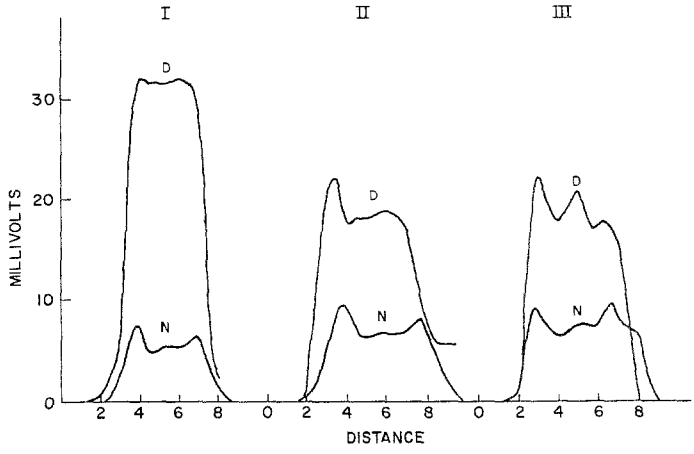


Fig. 7. Distribution of AChR activity before and after denervation for 7 weeks. The muscles were exposed to 2×10^{-5} M Carb for 2 min and the potential measured as the solution was drained. Distance along the muscle is in arbitrary units. I, II and III are three different frogs and *N* is the normal muscle and *D* the denervated muscle from each

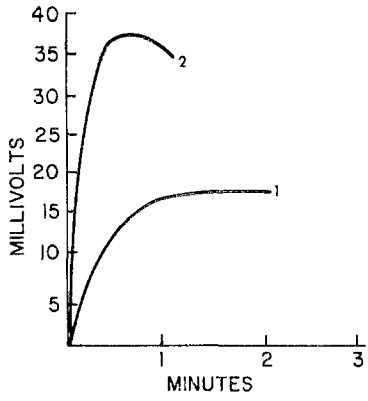


Fig. 8. Carb response after denervation for 6 weeks. Response to 2.5×10^{-3} M Carb (1) of a normal muscle, (2) of the contralateral denervated muscle

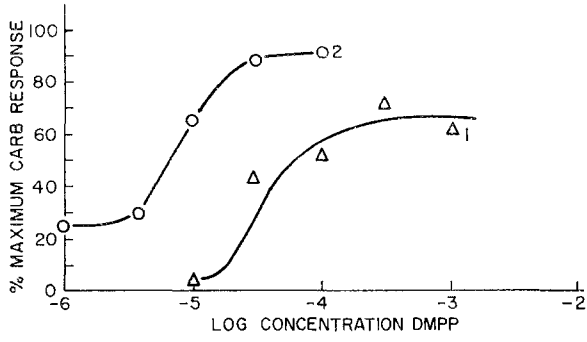
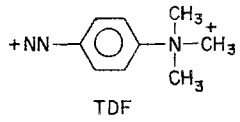


Fig. 9. Dose-response curves for dimethylphenylpiperazinium in (1) normal muscle, (2) contralateral muscle after denervation for 12 weeks. The response is expressed as a per cent of the maximum Carb response in each muscle

(n = 3) and an intrinsic activity of 0.85 ± 0.16 (n = 3). Typical dose-response curves from a normal and contralateral denervated muscle are shown in Fig. 9.

Effects of TDF on the Normal Response

If the tibial end of a sartorius muscle is exposed to 1×10^{-4} M TDF for 20 min at pH 7.0 and then rinsed exhaustively for up to 12 hr, the subsequent response to 2.5×10^{-3} M Carb is reduced by $74 \pm 13\%$ (n = 40). Higher concentrations of Carb do not increase the response. The concentration of agonist used to assay the irreversible blockage caused by TDF is important. For example, it is possible to block all response to 2.5×10^{-5} M Carb with 10^{-4} M TDF applied for 20 min while retaining 20% of the initial response to Carb at concentrations greater than 10^{-3} M. The response to 2.5×10^{-5} M



TDF

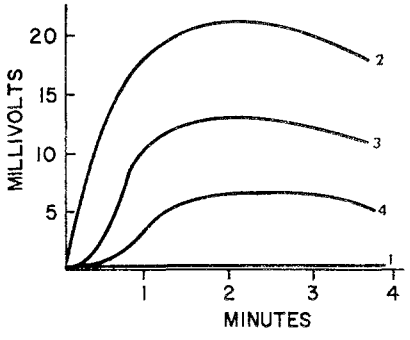


Fig. 10

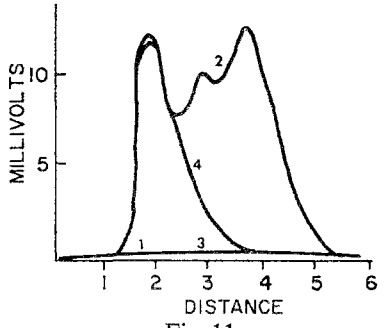


Fig. 11

Fig. 10. Blockage of the sartorius muscle Carb response by repeated doses of TDF. Response to (1) Ringer's solution alone, (2) 2.5×10^{-3} M Carb initially, (3) Carb, 1 hr after TDF at 4×10^{-5} M for 20 min (response is blocked 36%), (4) Carb, 1 hr after an additional 20 min TDF at 4×10^{-5} M (response is blocked 68%)

Fig. 11. Blockage of the AChR response in the tibial end of the sartorius muscle by TDF. The pattern of potential along the muscle is measured after (1) Ringer's solution alone, (2) Carb at 2.5×10^{-5} M applied for 9 min, (3) Ringer's, 1 hr after the tibial end of the muscle was exposed to TDF at 10^{-4} M for 20 min, (4) Carb at 2.5×10^{-5} M applied for 9 min, 1 hr after TDF

Carb is not blocked in direct proportion to the time of reaction with TDF, in contrast to the response to a concentration of Carb which gives a maximum response (2.5×10^{-3} M) (Fig. 10). The blockage caused by TDF is assayed with 2.5×10^{-3} M Carb in the following experiments on the hypothesis that the maximum Carb response is proportional to the total number of active AChR's present. This hypothesis is strongly supported by observations that the maximum response increases after denervation and decreases linearly with time after exposure to TDF, indicating that there are not AChR's in excess of those required for a maximum response. The amount of irreversible blockage produced by TDF decreases when thicker muscles are used, probably because of limited diffusion of TDF to the deeper end plates.

Exposure to TDF also results in extensive nonspecific modification of the muscle, as indicated by a faint yellowing of the tendon and the membrane overlying the muscle when TDF is used at concentrations of 10^{-4} or

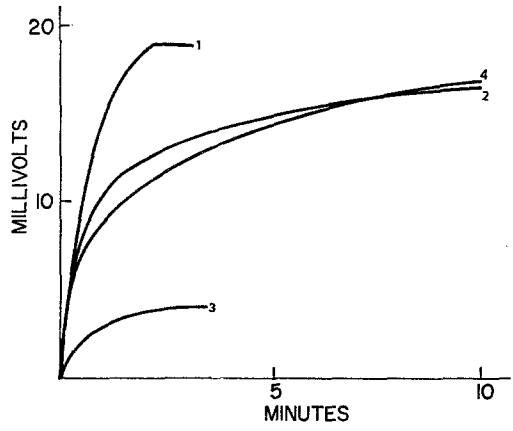


Fig. 12. Effect of blockage by TDF on depolarization by 30 mM K^+. The response is shown to (1) 2.5×10^{-3} M Carb, (2) 30 mM K^+ Ringer's solution, (3) Carb after TDF at 10^{-4} M for 20 min, (4) 30 mM K^+ Ringer's after TDF

above. If the pH is reduced to 6.0, the blockage caused by TDF is not reduced, but there is much less yellowing of the muscle. At pH 6.0, TDF at 1×10^{-4} M applied for 20 min blocks the maximum Carb response by $73 \pm 13\%$ ($n = 12$). Some evidence that the extensive reaction of TDF with the membrane at sites other than AChR's does not affect the responsiveness of the muscle is given in Fig. 11 which shows that even at pH 7.0, reaction with TDF does not depolarize the membrane. At high external potassium concentrations the sartorius muscle surface membrane behaves as a potassium electrode because the permeability to potassium is much higher than to other ions [1]. That reaction with TDF does not alter the ion selectivity of the membrane is indicated by the similar depolarization caused by 30 mM potassium before and after treatment with TDF (Fig. 12).

Reversibly bound AChR ligands can protect the AChR activity from irreversible blockage by TDF, strongly suggesting that TDF causes its irreversible blockage by reaction in the ACh binding site of the AChR. Although protection experiments are strongly suggestive, they cannot completely exclude the possibility that ACh binding sites, agonist, antagonist and TDF binding sites are distinct and mutually antagonistic through allosteric interactions. Protection is assayed using paired muscles from a single frog. On one of the muscles, the degree of blockage by TDF in the absence of protector is determined while on the contralateral muscle the degree of blockage by TDF in the presence of protector is measured. The tibial end of the protected muscle is preincubated with protector for 10 min

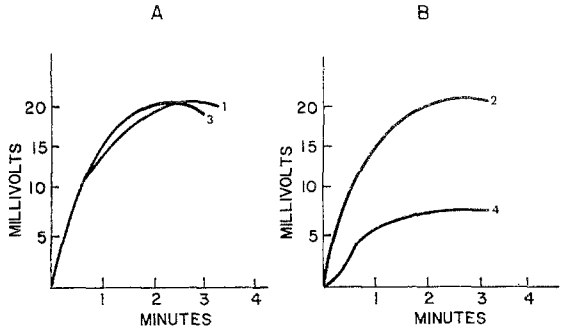


Fig. 13. Protection of TDF blockage by ACh. *A* and *B* are muscles from a single frog. *A* shows 100% protection. The response to 2.5×10^{-3} Carb: (1) initially in protected muscle, (2) initially in unprotected muscle, (3) after exposing the tibial end of the muscle to 2.5×10^{-2} M ACh, 5×10^{-5} M neostigmine for 10 min; then to ACh plus TDF at 10^{-4} M for 20 min; then to Ringer's solution for 3 hr, (4) after just neostigmine, TDF plus neostigmine, and Ringer's

before 10^{-4} M TDF plus protector is added for another 20 min. Then the muscle is exhaustively rinsed, often for several hours, to remove protector before both muscles are retested. Often, a third muscle from another animal is treated only with protector to determine whether blockage of the response is caused by residual protector. The per cent protection is defined as [1 −(% blockage in protected muscle/% blockage in unprotected muscle)] × 100%. After 20 min, TDF solutions with or without protector were assayed for unreacted TDF by reaction with *p*-cresol according to the method of Traylor and Singer [15], to determine if the concentration of TDF had been appreciably diminished by reaction directly with protector. Reaction of TDF with protectors or their counter ions was in fact a problem in the case of curare, benzoquinonium, nicotine, dimethylphenylpiperazinium, and iodide ion. These reactions limited the concentrations at which these reagents could be used as protectors or their counter ions. Reaction with curare was particularly extensive. Thus, when a solution containing a great molar excess of TDF over curare was made, the actual concentration of unreacted curare at any time was substantially below the initial concentration and substantial concentrations of the reaction products of TDF and curare were introduced.

Fig. 13 shows an experiment in which ACh gives 100% protection against irreversible blockage caused by TDF. High concentrations of protector may require many hours for complete removal during which the response may decay slightly, and prolonged exposure to high concentrations of protector may decrease the response of the muscle. Therefore, unless

responses are normalized to that of a muscle treated only with protector, it is often difficult to obtain apparent protection in excess of 80 to 90%.

Agonists, partial agonists, and antagonists all protect against the irreversible blockage of the Carb response caused by TDF. This is illustrated in Table 1. The degree of protection given by a particular concentration of any of these various ligands appears to be correlated with the fraction of AChR sites which are occupied at equilibrium by the ligand at that concentration in the absence of TDF. The fraction of sites occupied by a ligand is calculated using the equation, $a=A/(K_D+A)$ where a is the occupancy, A is the concentration of ligand, and K_D is its affinity constant. Regardless of the intrinsic activity of the protecting reagent, the per cent protection against TDF is approximately equal to the per cent occupancy of the AChR by the protector. Data for several protectors is plotted in Fig. 14. If the fractional response given by a concentration of agonist is equal to the equilibrium fraction of AChR that is bound (occupancy), then the fractional response given by a particular concentration should be equal to the fractional protection given by that concentration. Fig. 15 shows that this is approximately true for several concentrations of Carb. Protection in proportion to the apparent affinity of the protector is the result expected if there is one class of AChR site at which agonists, partial agonists, antagonists, and TDF act. These results further suggest that at 10^{-4} M, TDF does not compete for reversible binding to a significant extent. This implies that TDF has a very low reversible affinity for the AChR – probably less than or equal to that of phenyltrimethylammonium, $K_D=4\times10^{-4}$ M.

To determine whether reversible binding of the trimethylammonium group of TDF to the AChR was critical to the labeling reaction, as would be expected if TDF behaved as an affinity labeling reagent, the effects of several other aryldiazonium reagents were examined. If the quaternary nitrogen group of TDF were important to binding to the AChR, it might be expected that the uncharged analogue *p*-dimethylaminobenzene diazonium would have much lower affinity and therefore block much less [3]. However, this compound is intrinsically much less chemically reactive than TDF and so is not useful for examining this question. For example, unlike TDF, when mixed for 20 min at 10^{-4} M with 10^{-2} *p*-cresol in 1 M pH 6.0 phosphate buffer it does not react. Therefore, it is not significant that it does not block the AChR activity when applied at 10^{-4} M for 20 min. That the trimethylammonium group is not necessary for the reaction of TDF with the AChR is demonstrated by the fact that some other aryldiazonium salts which lack a trimethylammonium group are as effective as TDF in

Table 1. Protection of AChR against TDF

Protector	Activity	Apparent K_D	Concentration	Theoretical % occupancy of receptors at this concentration	% Protection against TDF at 10^{-4} M
Carb	agonist	2.7×10^{-5} M	10^{-5} M	27%	$29 \pm 2.5\%$ (n=4)
			3×10^{-5} M	53%	59% (n=1)
Tetramethylammonium	agonist	1.0×10^{-4} M	1×10^{-4} M	50%	45% (n=1)
ACh + 10^{-4} M neostigmine	agonist	1.5×10^{-6} M	10^{-6} M	40%	$29 \pm 1\%$ (n=2)
Propionylcholine + 10^{-4} M neostigmine	partial agonist	1.1×10^{-6} M	3×10^{-7} M	23%	$44 \pm 7\%$ (n=2)
Butrylcholine + 10^{-4} M neostigmine	partial agonist	1.0×10^{-6} M	3×10^{-7} M	23%	$29 \pm 6\%$ (n=3)
Nonanoylcholine	antagonist	1.4×10^{-7} M	1×10^{-5} M	99%	92% (n=1)
Dimethylphenylpiperazinium	partial agonist	2.8×10^{-5} M	10^{-5} M	26%	$27 \pm 7\%$ (n=2)
Nicotine	partial agonist	1.4×10^{-5} M	10^{-5} M	41%	35% (n=1)
Phenylthrimethylammonium	partial agonist	4.3×10^{-4} M	10^{-3} M	70%	80% (n=1)
HK 68 [7]	antagonist	2.0×10^{-4} M	1×10^{-4} M	33%	25% (n=1)
Curare	antagonist	3.8×10^{-7} M	10^{-5} M	100%	$29 \pm 2.7\%$ (n=5)

Paired muscles from a single frog were used for these experiments. The response to 2.5×10^{-3} M Carb of each muscle was determined. After washing to remove the Carb, one muscle was exposed to TDF at 10^{-4} M for 20 min while the other was pre-incubated with protector for 10 min and then exposed to TDF plus protector. After extensive washing to remove the protector, the response to Carb was again determined and the protection calculated as [1 − (% blockage in protected/% blockage in unprotected muscle)] × 100%. The apparent K_D of agonists was determined as the concentration giving a half maximal response, and the apparent K_D of antagonists was determined by competition with Carb. The theoretical per cent occupancy was caculated as $a = A/(K_D + A)$ where A is the concentration of ligand and K_D is the apparent affinity constant.

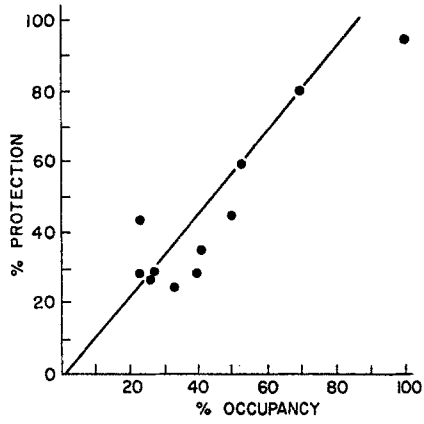


Fig. 14. Relationship between the protection against TDF and occupancy of AChR given by the agonists, partial agonists, and antagonists of Table 1. The occupancy calculated is that expected in the absence of TDF

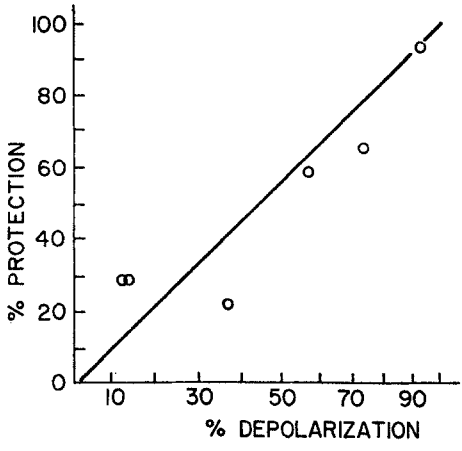


Fig. 15. Relationship between the degree of protection of the maximum Carb response against blockage by 10^{-4} M TDF and the response on that muscle by that concentration of Carb

blocking the AChR activity. PNBDF applied at 10^{-4} M for 20 min gives a blockage of $77.5 \pm 0.5\%$ (n=2). Reaction with PNBDF yellows the muscle and causes an irreversible nonspecific depolarization of about 17%. ACh at 10^{-5} M in the presence of 10^{-4} M neostigmine can protect 81% (n=1) against the blockage caused by 10^{-4} M PNBDF. MNBDF applied at 10^{-4} M for 20 min gives a blockage of $64 \pm 8\%$ (n=2), which is not accompanied by yellowing of the muscle or by irreversible depolarization. Thus, we would

conclude that the blockage of AChR activity by TDF does not depend on the reversible binding of TDF to the AChR due to affinity for the phenyltrimethylammonium group. It may be that the reaction of all these diazonium reagents involves reversible binding of the diazonium group to the anionic portion of the receptor site as has been suggested by Mautner and Bartels [10], but there is no reason to believe that the reaction is not simply a bimolecular reaction which does not require a reversible complex. The protection experiments demonstrated that if TDF does bind reversibly to the AChR, regardless of which cationic end is directed at the anionic portion of the site, it binds with very low affinity.

Effects of TDF on the Response of Denervated Muscle

The AChR response of denervated muscles is also blocked irreversibly by exposure to TDF. The blockage caused by exposure to low concentrations of TDF is about the same in normal and denervated muscles, and is approximately linear with time. For example, in a denervated muscle, TDF at 4×10^{-5} M for 20 min blocked 34.4%, and after an additional 20-min exposure to fresh TDF, 53.1%; whereas in a normal muscle the same treatments blocked by 36% and 68%, respectively.

The irreversible blockage of the AChR response in denervated muscles by TDF is protectable by reversibly bound ligands. For such protection experiments, the blockage by TDF in the presence and absence of protector is determined sequentially on the same muscle. This is a less accurate procedure than the protection experiment in which the blockage by TDF in the presence of protector is determined on the contralateral muscle because (1) it assumes linear rate of blockage by TDF on repeated exposures, (2) the TDF and protector concentrations must be chosen so that the sum of the blockage in the presence of protector and by TDF alone is sufficient to be measured accurately but not greater than 100%, and (3) such an experiment requires that the responses of the muscles be reproducible over many hours. If the fraction of AChR activity blocked on sequential exposure to TDF decreases slightly, the per cent protection estimated will be too low. On the other hand, if the response of the muscle decays over the duration of the experiment, the per cent protection will be an overestimate. It is possible to show essentially complete protection of the AChR activity from blockage by 10^{-4} M TDF in denervated muscles. ACh in the presence of neostigmine protects $88 \pm 9.0\%$ ($n=2$) at 10^{-5} M and Carb protects 39% ($n=1$) at 10^{-5} M against blockage by 10^{-4} M TDF. ACh in the presence of neostigmine

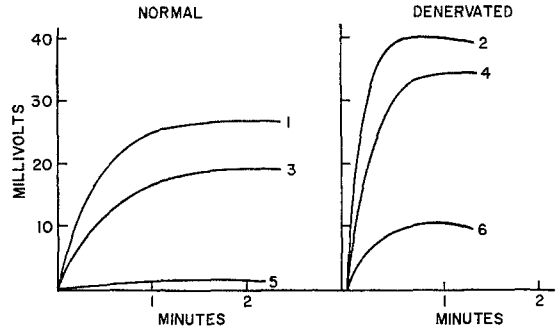


Fig. 16. Protection of AChR against blockage by TDF in a normal and contralateral muscle denervated for 7 weeks. The initial responses to 2.5×10^{-3} M Carb are shown by (1) and (2). The response, 4 hr after 10-min pretreatment with 10^{-6} M ACh 10^{-4} M neostigmine followed by 20 min with ACh plus 10^{-4} M TDF is shown by (3) and (4). And the responses 1 hr after 20 min of TDF applied 30 min after (3) and (4) are shown by (5) and (6). The normal muscle shows 52% protection and the denervated muscle, 73% protection

protects 73% at 10^{-6} M (n = 1) against 5×10^{-5} M TDF. This experiment is illustrated in Fig. 16.

Conclusions

TDF reacts irreversibly with the AChR in its ACh binding site thereby irreversibly blocking the response of the sartorius muscle to agonists. Irreversible blockage of the AChR by TDF can be prevented by the presence of appropriate concentrations of agonists, partial agonists, and antagonists which compete with TDF for occupancy of the ACh binding site of the AChR. A given concentration of any of these ligands protects the AChR from irreversible blockage by TDF by a fraction equal to the theoretical fractional occupancy of AChR sites for that ligand concentration calculated from the apparent affinity constant of the ligand for the AChR. This indicates that in frog sartorius muscle there is one type of ACh binding site at which all ligands act regardless of their intrinsic activity. It also indicates that in this case it is a valid approximation to assume that the fraction of the maximum response given by a particular concentration of agonist or partial agonist is equal to the fraction of the AChR's occupied. This assumption is also supported by the demonstration that AChR's are not present in excess of those required for a maximum response. Because there is evidence that partial agonists do not alter the selectivity of the permeability

change caused by their interaction with AChR (which would alter the reversal potential) [8], the observation that partial agonists bind to the same sites as agonists suggests that the binding of a partial agonist molecule may cause a smaller increment of permeability than the binding of an agonist molecule during the time the receptor is activated.

Protection experiments demonstrate that TDF has very low reversible binding affinity for the sartorius muscle AChR. This might be expected because phenyltrimethylammonium, which is structurally analogous to TDF, also has very low affinity. Its low affinity makes TDF a useful tool for studying some aspects of AChR activity. However, it is questionable whether TDF should be thought of as an affinity labeling reagent for this AChR activity because it is difficult to demonstrate that its reaction is dependent upon affinity for the AChR. It could be that TDF irreversibly blocks the AChR through a simple bimolecular reaction. Two other diazonium reagents, MNBDF and PNBDF, which lack a trimethylammonium group, are as effective as TDF in irreversibly blocking the AChR through reaction in the AChR binding site. It could be, as Mautner and Bartels [10] have suggested, that this is a result of reversible binding of the cationic diazonium group to the AChR, but it is not necessary to suppose that these reagents must be reversibly bound to react. That dimethylaminobenzene diazonium is much less reactive than TDF because it lacks an electron withdrawing group *para* to the diazonium group as is present in both *p*-nitrobenzene diazonium and TDF (*p*-trimethylammoniumbenzene diazonium) has been suggested by Mautner and Bartels [10]. This explanation is consistent with our results and would account for the somewhat lower extent of reaction by *m*-nitrobenzenediazonium.

Because of the low reversible binding affinity of TDF for the AChR, TDF must be used at concentrations which result in such extensive nonspecific reactions that a pale yellow coloration of the muscle is produced. Nevertheless, all of the irreversible blockage of the AChR activity is caused by reaction in the ACh binding site of the AChR as shown by protection experiments. Furthermore, the depolarization caused by high concentrations of potassium is not significantly altered by these extensive nonspecific reactions. That the extent of nonspecific reaction with TDF is very considerable was also demonstrated by experiments with ^{3}H-TDF on sartorius and rat diaphragm muscles[3]. ^{3}H-TDF at concentrations between 10^{-6} and 10^{-4} M was reacted with sartorius muscles or strips of diaphragm muscle.

3 Unpublished observations.

The muscles were rinsed exhaustively, cut into small sections and the ^{3}H-TDF bound per weight of tissue determined. ^{3}H-TDF bound over the whole muscle with no increase in binding in the end plate region. Binding was actually highest in the tendons. Neither ACh, curare, nor flaxedil applied with the ^{3}H-TDF decreased the amount of binding, nor did denervation increase the binding or affect its distribution. Evidently, the AChR sites comprise only a small fraction of the total sites at which TDF reacts. Thus, ^{3}H-TDF is not an adequately specific labeling reagent to attempt to specifically label AChR molecules in these muscles.

Since phenyltrimethylammonium has higher affinity for the AChR of electroplax than sartorius muscle, it might be expected that TDF also would have higher affinity for the electroplax AChR and thus show greater specificity. However, since the exposure to TDF required for substantial blockage to occur is very similar with both tissues, it seems likely that TDF does not have substantially greater affinity for the electroplax AChR and would not react there with significantly greater specificity.

It is interesting to consider the degree of specificity which would be required of an affinity labeling reagent so that a significant fraction of the sites at which it reacted were AChR's. When TDF is reacted with purified anti-phenyltrimethylammonium antibodies present in nearly equal molar concentration, about 5% of the labeling occurs at nonspecific sites which cannot be protected [5]. Other diazonium affinity labeling reagents for specific anti-hapten antibodies with affinity constants of 10^{-4} to 10^{-5} M also label about 5% nonspecific sites under these conditions [13]. When affinity labeling reagents are used to label receptor sites on membranes, however, the specific receptor protein is surrounded by a great excess of other proteins and other reactive compounds and the labeling reagent is present in great molar excess. It is reasonable to suppose that the receptor might comprise 0.1% of the membrane protein, or even less. If such a 1,000-fold excess of nonspecific protein were added to the reaction mixtures with the specific antibody, the rate of nonspecific reaction would increase 1,000-fold so that 98% of the labeling would be nonspecific. Of course, if the antibody protein could be resolved from the nonspecific protein, the 2% of the label associated with it would be 95% protectable. Resolution of that small a component of specifically labeled receptor from other membrane components would be very difficult. Also, the problem could be complicated by slight affinity enhancement of reaction rate or protection at nonspecific sites present in higher concentrations than the receptor. Higher specificity can be achieved by labeling reagents with higher reversible binding affinity,

because, if the reactive group is reversibly bound in an orientation which allows it to react, the specificity increases in proportion to the reversible binding affinity [13]. The reversible binding affinity required for a highly specific affinity labeling reagent is inversely proportional to the concentration of the receptor site, and for the AChR is probably extremely high. If that affinity can be achieved by structural modifications of the labeling molecule that do not also greatly increase its nonspecific binding to, for example, hydrophobic or anionic regions of the membrane, then the labeling reagent will also be much better able to discriminate among the related nonspecific binding sites such as ACh esterase and other cation-binding sites which may be present [14].

Chronic denervation causes the response induced by Carb to increase in absolute value, and to develop and desensitize more rapidly. Denervated muscles respond to much lower concentrations of agonists, often giving a half maximal response at 1/10th the concentration normally required. After denervation, increased numbers of AChR's are thought to be formed at areas outside the nerve-muscle junction [11]. The denervated muscles used in this study were not uniformly sensitive over their entire surfaces, although perhaps substantial spreading of sensitivity could have occurred.

Of interest is whether the new AChR's formed after denervation have the same properties as those present before denaturation. The response measured in this assay system does not provide sufficient information for a detailed electrophysiological interpretation of differences in AChR activity before and after denervation. Certainly the AChR activity after denervation has several features qualitatively in common with normal activity. The AChR activity after denervation is blocked by the irreversible inhibitors TDF, NEM [9], and MPTA [9]. As in the normal muscle, blockage by TDF of the maximum Carb response shows no lag period, indicating that there are not AChR's in excess of those required for a maximum response. And the irreversible blockage by TDF is protectable by agonists. However, denervated muscles give a half maximal response to concentrations of agonists as much as 10-fold lower than normal, and the apparent intrinsic activity of decamethonium and dimethylphenylpiperazinium increases. It is possible that this reflects either real differences in the microscopic properties of the AChR formed after denervation or that the response measured by this assay differs artifactually from normal as the result of high numbers of normal AChR's acting in atrophied muscle fibers. One way to resolve this might be extensive protection experiments, using contralateral denervated muscles to determine the relationship between occupancy and protection as was done for normal

muscles. Then, for example, if concentrations of agonists which gave very low responses and protection of the maximum response in normal muscles gave both very large responses and very large protection in denervated muscles, it would seem likely that dose-response curves obtained with denervated muscles resulted from AChR having higher affinity for agonists.

In addition to TDF, two other types of irreversible inhibitors of the AChR activity of frog sartorius muscles have been studied. Studies with sulfhydryl alkylating reagents will be reported in a subsequent paper [9]. In a previous paper [7], we described the use of a photolyzable affinity labeling reagent which is a poor antagonist in the dark and irreversibly blocks the AChR through reaction in the ACh binding site after photolysis with ultraviolet light.

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