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The Effects of Reducing and Alkylating Agents on the Acetylcholine Receptor Activity of Frog Sartorius Muscle

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Summary. Exposure of frog sartorius muscle to the disulfide bond reducing agent, dithiothreitol, caused a decrease in the apparent affinity of their acetylcholine receptors for some agonists. Exposure to the oxidizing agent $K_3Fe(CN)_6$ reversed this effect. After reduction, the response to agonists was irreversibly blocked by exposure of the muscles to N-ethyl maleimide or 4-(N′-maleimido)phenyltrimethylammonium. However, contrary to what had been observed with electroplax, the blocking reaction did not occur at the acetylcholine binding site of the receptor.

The remarkable effects of sulfhydryl reagents on the AChR[1] activity of eel electroplax have been extensively studied by Karlin and his associates [3–5, 9], who showed that exposure to the disulfide bond reducing reagent DTT diminished affinity of the electroplax AChR for Carb, and altered the intrinsic activity of hexamethonium from that of antagonist to agonist. These effects were reversed by exposure of the DTT-treated electroplax to oxidizing agents. This suggested that DTT reduced a disulfide bond (or bonds) which caused an alteration in the conformation of the AChR molecule. If reduced electroplax were exposed to the sulfhydryl alkylating reagent NEM, the response to Carb could no longer be restored by oxidation, which suggested the AChR was irreversibly altered by alkylation of sulfhydryl groups formed by reduction of the critical disulfide bond. On the hypothesis that this critical disulfide bond was located in the ACh

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1 Abbreviations used are: AChR, acetylcholine receptor; DTT, dithiothreitol; Carb, carbamylcholine; NEM, N-ethyl maleimide; MPTA, 4-(N′-maleimido)phenyltrimethylammonium; MBTA, 4-(N′-maleimido)benzyltrimethylammonium; TDF, *p*-trimethylammonium benzene diazonium difluoroborate.

binding site of the receptor, Karlin and Winnik [5] synthesized a maleimide reagent MPTA which, like ACh, contained a quaternary ammonium group. MPTA behaved as a reversible antagonist of the unreduced electroplax AChR; but irreversibly blocked the reduced AChR at a 1,000-fold greater rate than did NEM. This suggested that specific reversible binding of MPTA to the ACh binding site of the receptor enhanced the rate of its reaction with a sulfhydryl group formed in or near that site by reduction. In further support of this interpretation, AChR activity of reduced electroplax could be protected from blockage by MPTA with the reversible AChR ligand hexamethonium. Hence, in the electroplax, MPTA exhibited the two critical properties expected of an affinity labeling reagent [10], affinity enhancement of reactivity, and, by the criterion of protection experiments, labeling in the active site.

We report investigations on the effects of both reducing and alkylating agents on AChR activity of frog sartorius muscle. We found that although several of the effects of these agents were similar to those observed in electroplax, including blockage of reduced sartorius muscle AChR activity by alkylating agents, this blockage did not result from alkylation in the ACh binding site of the receptor. Thus, MPTA could not be used as an affinity labeling reagent in this preparation.

Materials and Methods

The AChR activity of frog sartorius muscle was assayed by a modification of the fluid electrode technique of Fatt [2] described previously [6], except that the Ringer's solution in which the muscles were bathed was buffered with 3 mM Tris, pH 8.0. At this pH the maximum Carb response was $31 \pm 8\%$ ($n=2$)[2] lower than with phosphate buffer at pH 7.0. For these assays, a muscle was suspended from its pelvic end in a cylinder and a wick electrode placed at the pelvic end, which lacks end plates. Solutions were introduced into the cylinder from the bottom so that they contacted the tibial end of the muscle first. When a Ringer's solution containing agonist was introduced into the cylinder so that the meniscus was located on a part of the muscle containing end plates, a potential difference developed between the pelvic electrode and the bath electrode. Experiments were performed in two ways. In one method, the meniscus was kept at a fixed point on the muscle containing a high concentration of end plates and the change of potential with time was recorded (Figs. 2 through 7). In the second method, the whole muscle was bathed with agonist solution and at intervals the solution was rapidly drained so that the meniscus swept down the surface of the muscle allowing recording of the potential at each point along the muscle as the meniscus contacted it. The moving meniscus method thus indicated both the distribution and degree of response at a fixed time (Fig. 1).

MPTA was synthesized by H. Kiefer using the methods of Karlin and Winnik [5]. MBTA was a gift from A. Karlin.

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

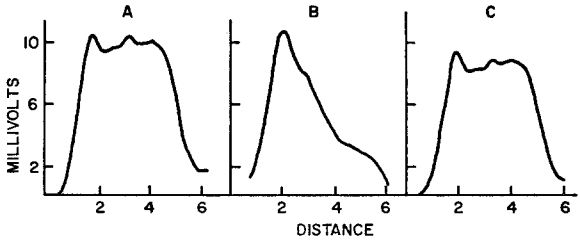

Fig. 1. Effects of DTT and $K_3Fe(CN)_6$ on the ACh response. (*A*) Shows the distribution of depolarization along a muscle after 12-min exposure to 2×10^{-6} M ACh, 10^{-5} M neostigmine. (*B*) Shows the response after 5 min of exposure of the tibial end of the muscle to 10^{-3} M DTT. The response is again measured after 12-min exposure to ACh. (*C*) Shows that the response in the tibial end is restored by exposure to 10^{-3} M $K_3Fe(CN)_6$ for 5 min. The response at the pelvic peak is an internal control for the responsiveness of the muscle, indicating that the response of untreated portions of the muscle is virtually unaltered during the time required for the experiment

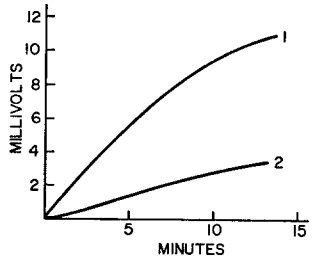

Fig. 2. Inhibition of the response to 2.5×10^{-5} M Carb by DTT. The initial response to Carb applied at a fixed point on the muscle is shown in (1) and the response obtained after treatment with 10^{-3} M DTT for 20 min is shown in (2). The response is decreased 68%

Results

Exposure of sartorius muscle to the reducing agent DTT inhibited the response to low concentrations of agonist, but only slightly reduced the response to high concentrations of agonist. This was similar to the result obtained by Karlin and Bartels [4] with the monocellular electroplax preparation, and suggested that the affinity of receptor for agonists was decreased by the DTT treatment. Exposure of muscles to 10^{-3} M DTT for 5 min inhibited the response to 2×10^{-6} M ACh by $48 \pm 11\%$ ($n = 7$) (Fig. 1). Increasing the exposure to 20 min increased the degree of blockage to only 68%. Since exposure to 10^{-3} M DTT for 20 min gave a near maximum effect, this treatment was used routinely in subsequent experiments and simply referred to as "reduction". The response to 2.5×10^{-5} M Carb was also blocked by 68% after reduction (Fig. 2). However, the maximum response (assayed with 2.5×10^{-3} M Carb) was decreased by only $5.3 \pm 5.0\%$ ($n = 18$).

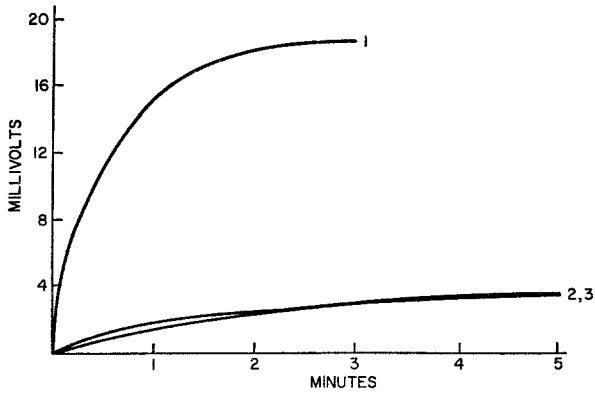

Fig. 3. Effect of DTT on the response to decamethonium. (1) Shows the response to 2.5×10^{-3} M Carb at a fixed point on the muscle. (2) Shows the response to 10^{-4} M decamethonium. (3) Shows the response to decamethonium after exposure of the tibial end of the muscle to 10^{-3} M DTT for 20 min. The response is not significantly altered

Karlin [3] observed that reduction increased the response of electroplax to low concentrations of decamethonium, but did not examine the effects of reduction on the maximum response to decamethonium. Since in electroplax the maximum response to decamethonium is normally somewhat lower than that to Carb [1], this observation could have resulted from an increase in the affinity of receptor for this agonist (as opposed to the decrease in affinity observed with other agonists) and/or from an increase in intrinsic activity of decamethonium (as observed with hexamethonium). The maximum response of frog sartorius muscle to decamethonium is normally less than half that of Carb or ACh [6], so that an increase in intrinsic activity of decamethonium would be easily detected as an increase in the maximum response to decamethonium. It was found that reduction did not alter the maximum response to decamethonium (Fig. 3). The effects of reduction on the response to lower concentrations of decamethonium were not examined.

As in the electroplax [3], the effects of reduction on the response of sartorius muscle could be completely reversed by exposure of the muscle to the oxidizing agent $K_3Fe(CN)_6$ (Fig. 1). Blockage of the response to low concentrations of Carb caused by reduction was also spontaneously reversed over several hours, probably as a result of gradual air oxidation.

Although sartorius muscle was not normally depolarized by exposure to the irreversible inhibitor TDF, after reduction, treatment with TDF caused depolarization. However, after reduction, irreversible blockage of the Carb response by TDF was not diminished. Depolarization of reduced muscles by 10^{-4} M TDF occurred at a much slower rate than expected for a

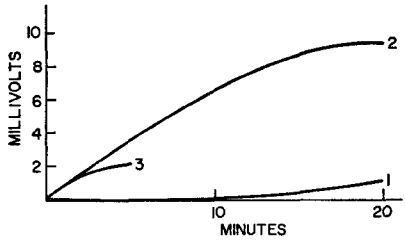

Fig. 4. Depolarization caused by TDF in muscles exposed to DTT. (1) Shows the negligible amount of depolarization caused by 10^{-4} M TDF maintained at a fixed point on the muscle. (2) Shows that after exposing a muscle to 10^{-3} M DTT for 20 min, 10^{-4} M TDF caused a depolarization of the muscle to develop. (3) Shows the depolarization caused by 10^{-4} M phenyltrimethylammonium in a normal muscle to illustrate the slow time course of the depolarization by TDF

reversible agonist at that concentration (Fig. 4). This depolarization was also only slowly reversed by washing, and 25% remained after 4 hr. This depolarization was probably caused by covalent reaction of TDF at sites other than the ACh binding site of the receptor. This interpretation was suggested by the observations that TDF retained its blocking activity, that the depolarization occurred very slowly, and that TDF was known to react extensively with the surface of sartorius muscle before reduction [6]. These results differed from those with electroplax [1, 7] where, after reduction, TDF behaved as a reversible agonist.

After reduction, exposure of muscles to NEM irreversibly blocked the response to Carb, whereas prior to reduction, NEM had little effect. This was similar to the effect of NEM on the electroplax [3]. The blockage caused by alkylation was measured by the decrease in the maximum Carb response (which was essentially unaffected by reduction alone). Since the maximum Carb response was approximately proportional to the total number of active AChR sites [6], this procedure gave a direct estimate of the fraction of receptors inactivated by alkylation. Exposure of a normal muscle to 10^{-4} M NEM for 5 min had no effect on the maximum response, although a 20-min exposure produced a 10% decrease in the response, accompanied by impaired repolarization. After reduction, exposure to 10^{-4} M NEM for 20 min caused a $39 \pm 6.1\%$ ($n=4$) blockage of the maximum response. This blockage was not reversed by $K_3Fe(CN)_6$.

Prior to reduction, MPTA was a low affinity reversible antagonist ($K_D = 3.8 \pm 2.0 \times 10^{-4}$ M ($n=5$)) and did not exhibit the nonspecific effects of NEM. After reduction, MPTA blocked the maximum response to Carb (Figs. 5 and 6). As expected of an alkylating agent, this blockage could not be reversed by oxidation (Fig. 5). Blockage by MPTA did not alter the

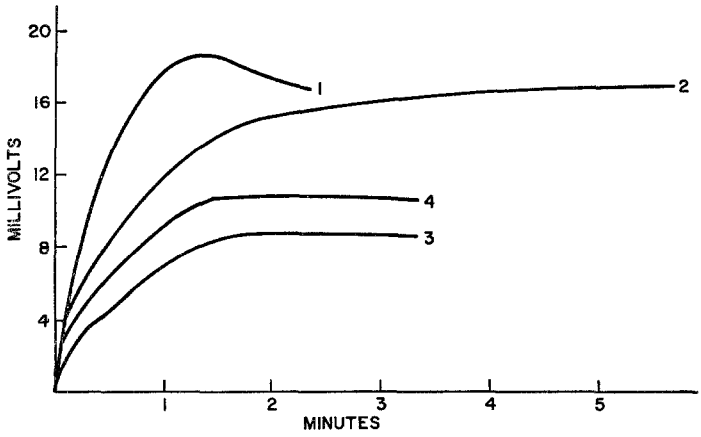

Fig. 5. Effects of DTT and MPTA on the response to Carb. (1) Shows the response to 2.5×10^{-3} M Carb at a fixed point on the muscle. (2) Shows the response after 20-min exposure of the tibial end of the muscle to 10^{-3} M DTT. When corrections are made for the potential initially present at that point, the response is 96% of (1). (3) Shows the response after DTT followed by 20-min exposure to MPTA at 10^{-4} M. The response is 52% of (1). (4) Shows the response after DTT and MPTA followed by exposure to $K_3Fe(CN)_6$ at 10^{-3} M for 20 min. The response is 62% of (1)

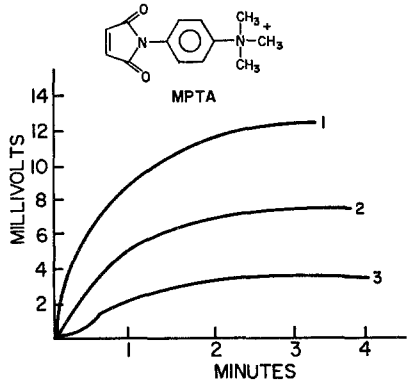

Fig. 6. Blockage of Carb response by MPTA after treatment with DTT. (1) Shows the response to 2.5×10^{-3} M Carb at a fixed point on the muscle. (2) Shows the response to Carb after DTT at 10^{-3} M for 20 min, Ringer's solution for 10 min, MPTA at 10^{-4} M for 20 min, Ringer's solution for 20 min. (3) Shows the response to Carb after this sequence of exposure to DTT and MPTA was repeated

depolarization caused by 30 mM K^+ in Ringer's solution. At 10^{-4} M, MPTA applied for 20 min to a reduced muscle blocked the response by 43 ± 7.8% (n = 4). Repeated or prolonged exposure to MPTA resulted in further blockage (Fig. 6) which could be continued until all response was abolished. At 10^{-5} M, MPTA did not produce detectable blockage. Thus, MPTA was no more potent an alkylating agent than NEM. Varying the pH of the

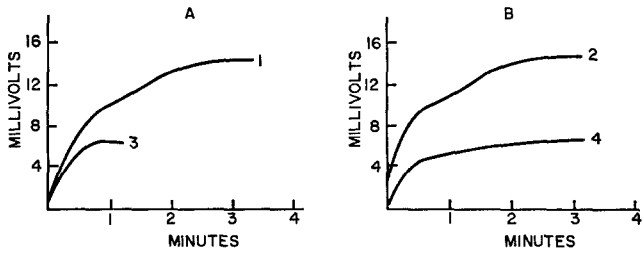

Fig. 7. Failure of curare to protect against MPTA. (1) and (2) Show the initial response to 2.5×10^{-3} M Carb at a fixed point on contralateral muscles. (3) Shows the response 3 hr after DTT at 10^{-3} M for 20 min, Ringer's solution for 20 min, MPTA at 10^{-4} M for 20 min. (4) Shows the response 3 hr after DTT, Ringer's solution for 10 min, curare at 10^{-5} M for 10 min, curare plus MPTA for 20 min. All solutions except the Ringer's solution rinses were applied only to the tibial half of the muscles. Muscle *A* showed 54% block; muscle *B* showed 55% block. Deviation from a smooth curve as shown in (1) and (2) was occasionally observed and was probably the result of slowed diffusion of Carb to end plates in unusually thick areas of the muscles

Ringer's solution did not greatly alter the potency of MPTA. Blockage by a 20-min exposure to 10^{-4} M MPTA increased from 30% to 50% between pH 7.0 and 9.0. These results differed from those in the electroplax [3] where MPTA was 1,000-fold more potent than NEM.

To determine if MPTA failed to show the expected affinity enhancement of reactivity because the maleimido group was not properly oriented in the binding site, the effects of MBTA were assayed. Karlin [3] observed that MBTA was even more effective than MPTA in the electroplax. A 20-min exposure to 10^{-4} M MBTA blocked only 39% of the maximum response, and therefore was no more potent than NEM or MPTA. Thus, the reaction of neither MPTA nor MBTA was enhanced by specific reversible binding to the AChR.

Blockage of the Carb response by NEM, MPTA or MBTA could not be prevented by agonists or antagonists (Fig. 7 and Table 1). Ligands were used to protect the AChR at concentrations which gave significant protection against irreversible inhibition by TDF [6] and with which it was expected that virtually all of the AChR sites were occupied by the ligand molecules. It was also observed that the AChR was not protected from the effects of reduction (as assayed by the blockage of the response to low concentrations of Carb) by 10^{-5} M curare. These results contrasted with the observation that blockage of the electroplax AChR could be prevented by high concentrations of hexamethonium. Failure of active site ligands to protect the AChR from these agents strongly suggested that NEM, MPTA and MBTA did not alkylate the ACh binding site of the sartorius muscle AChR.

Table 1. Failure of ligands to protect against blockage by MPTA

Protector	Concentration	% Protection against 10^{-4} M MPTA
Curare	10^{-5} M	0 (n=3)
Carb	2.5×10^{-2} M	0 (n=1)
Nonanoylcholine (10^{-4} M neostigmine)	10^{-5} M	0 (n=1)
Decanoylcholine (10^{-4} M neostigmine)	10^{-5} M	0 (n=2)

Paired muscles were used in these experiments. After reduction, one was exposed only to MPTA, whereas the other was pretreated with the protector for 10 min and then exposed to MPTA plus the protector. Zero protection indicates that the response to 2.5×10^{-3} Carb was equally blocked in both muscles.

The effects of NEM and MPTA on the Carb response of chronically denervated muscles were also examined. The maximum Carb response of these muscles was blocked, but to a slightly lesser extent than was the response of normal muscles. After reduction, 10^{-4} M NEM blocked 31 ± 11 % (n=3) in 20 min. Under similar conditions, MPTA blocked 24 ± 1.5 % (n=2).

Discussion

The effects of reduction on the AChR activity of frog sartorius muscle showed certain similarities to the effects of reduction on electroplax [4] or chicken muscle [8]. Reduction of a disulfide bond (or bonds) in the postsynaptic membrane in or near the AChR macromolecule decreased the apparent affinity of the AChR for Carb and ACh. Reduction decreased the response to low concentrations of agonist to an extent consistent with a fivefold decrease in the affinity of AChR for these ligands. Although reduction increased the response of electroplax to low concentrations of decamethonium, and increased the intrinsic activity of hexamethonium, reduction did not increase the intrinsic activity of decamethonium on sartorius muscle. Effects of reduction may depend critically on the structure of the ligand used. For example, Rang and Ritter [8] observed that reduction did not alter the response of chicken muscle to decamethonium, but it increased the apparent affinity for the corresponding 7-carbon chain length compound ninefold, while it decreased the affinity for the corresponding 2-carbon compound sixfold.

Although the irreversible inhibitor TDF behaved as a reversible agonist on the reduced electroplax [1, 7], it did not diminish in irreversible blockage

of the reduced sartorius muscle AChR. After reduction, TDF depolarized sartorius muscle with an unusually slow time course which suggested that this depolarization was the result of TDF reacting at sites other than the AChR. TDF is known to undergo extensive nonspecific reaction with sartorius muscle [6].

After reduction, NEM irreversibly blocked the maximum Carb response. This suggested that in sartorius muscle, as in electroplax, thiol groups affecting the AChR could be alkylated by NEM. In sartorius muscle the maximum response to Carb, which was virtually unaffected by reduction, was blocked by alkylating agents, indicating that although reduction altered the binding of agonists, alkylation completely prevented either the binding of agonists or the transduction of binding into a permeability change.

On normal muscles, MPTA behaved as a poor reversible antagonist, with about fivefold lower affinity than for the electroplax AChR. The lower affinity was not unexpected since sartorius muscle AChR showed lower affinity for phenyltrimethylammonium than did electroplax [6]. The affinity of sartorius muscle AChR for MPTA and phenyltrimethylammonium was nearly equal.

After reduction, both MPTA and MBTA irreversibly blocked the maximum Carb response, but these agents were no more effective than NEM. If their site of reaction were in or very near the ACh binding site of the receptor, specific reversible binding of these agents would be expected to greatly enhance their reaction rate. Affinity enhancement of reactivity occurred with the electroplax [3] where MPTA blocked AChR activity 2,500-fold faster than NEM (only 460-fold when the enhanced reactivity of MPTA with cysteine was accounted for) and MBTA blocked AChR activity 4,650-fold faster than NEM. Even though the frog sartorius showed somewhat lower affinity for MPTA, substantial enhancement of reaction rate would be expected if the reaction of MPTA depended on reversible binding to the AChR. Karlin showed [3] that MPTA and MBTA were 5.5- and 4.3-fold, respectively, more reactive with cysteine than NEM; thus the equal blockage given by NEM, MPTA and MBTA in the frog sartorius implied that the phenyltrimethylammonium group in MPTA and MBTA actually reduced their effectiveness with respect to NEM.

Protection experiments strongly suggested that alkylation did not occur in the ACh binding site. The AChR could not be protected from blockage by agonists or antagonists. Nor could the AChR activity be protected from the effects of reduction by curare. It has also been reported that chicken muscle AChR cannot be protected from reduction by curare [8]. For

alkylation to have occurred in the ACh binding site at an undiminished rate in the presence of a protector, the alkylating agent would have had to continue to occupy all of the AChR sites. This could have occurred only if the alkylating agent were bound with very great affinity, which was incompatible with the observed lack of affinity enhancement of reactivity. Therefore, alkylation must have occurred at amino acid residues outside of the ACh binding site of the receptor. Alkylation could have occurred on the AChR macromolecule near the ACh binding site, near the membrane permeability controlling portion of the AChR, or on structures near the AChR which affected its activity.

After reduction, the AChR activity of denervated muscles was blocked by NEM and MPTA, although somewhat less effectively than was the AChR activity of normal muscles.

Although MPTA and related alkylating agents irreversibly blocked the activity of the acetylcholine receptor of frog sartorius muscle, both before and after denervation, these agents could not be used for specific radioactive labeling of this receptor because they did not act as affinity labeling reagents.

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