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The Effect of Disulfide Bond Reduction on the Properties of Cholinergic Receptors in Chick Muscle

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SUMMARY

The effect of treatment with the disulfide bond-reducing agent dithiothreitol on the responsiveness of isolated strips of chick muscle to cholinergic agonists and antagonists has been studied. After reduction, the muscle became less sensitive to carbachol, acetylcholine, and several other monoquaternary agonists, but more sensitive to bisquaternaries of the decamethonium series with chain lengths from C_6 to C_9. Hexamethonium, normally a weak antagonist, became an agonist. The relation between chain length and potency within two homologous series was only slightly modified by reduction of the receptors. The effects of reduction could be fully reversed by reoxidation with 5,5′-dithiobis(2-nitrobenzoic acid). The stimulant actions of caffeine and potassium ions were unaffected by reduction. Tubocurarine did not protect the receptors against the action of dithiothreitol.

Reduction increased the affinity of tubocurarine for the receptors, by a factor of 2.4. It also greatly increased the blocking potency of the alkylating agent diphenyldecamethonium mustard and, to a smaller extent, that of the corresponding dinaphthyl compound.

These results, which are comparable to those obtained on electroplax cells, suggest that the receptor possesses a disulfide bond, reduction of which modifies the selectivity of the receptor without markedly affecting its function.

INTRODUCTION

These have been a number of studies of the effects of reduction of tissue disulfide bonds, and of sulfhydryl-blocking agents, on drug action at the cellular level [for references, see Ehrenpreis *et al.* (1)]. The most detailed studies have been those of Martin and Schild (2) on the actions of peptides related to oxytocin and vasopressin, end of Karlin and his associates (3–7) on the actions of depolarizing and blocking drugs on electroplax cells from *Electrophorus electricus*. One of the most striking effects was that the depolarizing action of carbachol was greatly inhibited after the cell had been treated with dithiothreitol (8), whereas hexamethonium, which lacks depolarizing activity against a normal cell, caused depolarization of the dithiothreitol-treated cell (4). These effects were fully reversed by exposure to the oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid) (9). Karlin and his associates also found that the susceptibility of the receptors to alkylation by quaternary ammonium derivatives of *N*-ethylmaleimide was increased by dithiothreitol treatment (4, 6). On the basis of these findings, Karlin (6) proposed that the receptor for carbachol is a protein containing a disulfide bridge in the neighborhood of the active site, reduction of which exposes sulfhydryl groups that are readily attacked by certain alkylating agents.

In this study we have examined the effects

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of dithiothreitol and 5,5′-dithiobis(2-nitrobenzoic acid) on the reactivity of chick muscle to cholinergic agonists and antagonists, in order to characterize in more detail the effect of disulfide bond reduction on the receptors in this tissue, and to compare it with the electroplax.

A preliminary account of this work has been published (10).

METHODS

The experiments were done on thin strips of chick biventer cervicis muscle prepared and mounted in Krebs' solution, pH 7.4, at 37°, as described previously (11).

Solutions of $DPC_{10}M$[1] were incubated at neutral pH to generate the active ethyleniminium ion as described earlier (11). $DNC_{10}M$ was made up as a 1 mM stock solution in 0.01 N nitric acid containing 10% ethanol. This was diluted to 0.4 mM by adding 0.07 M sodium phosphate buffer (pH 7.4) and kept at 37° for 30 min. This procedure gave a 70% yield of the ethyleniminium ion, estimated by titration with thiosulfate; the ethyleniminium ion was stable for some hours if the solution was kept on ice. Concentrations of $DPC_{10}M$ and $DNC_{10}M$ are expressed in terms of the active ethyleniminium ion.

Dithiothreitol (Calbiochem) and DTNB (British Drug Houses) were applied in Krebs' solution (pH 7.4). In the electroplax at room temperature it has been reported (3, 4) that these reagents are effective if applied at pH 8, but not at pH 7. Since, in our experiments, DTT and DTNB were found to be fully effective at physiological pH, we did not investigate the pH dependence of the reactions.

[1] The abbreviations used are: DPC_{10}, decamethylene - 1,10 - bis(dimethylbenzylammonium bromide); $DPC_{10}M$, decamethylene-1-(*N*-benzyl-2-chloroethylamino) - 10 - dimethylbenzylammonium chloride hydrochloride; $DNC_{10}M$, decamethylene-1 - (2 - chloroethyl - 1 - naphthylmethyl) - amine - 10-dimethyl-(1-naphthylmethyl)ammonium chloride hydrochloride; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); C_n-TMA, alkyltrimethylammonium compounds of the type $CH_3(CH_2)_{n-1}N^+(CH_3)_3$; C_n-bis-TMA, alkylbis (trimethylammonium) compounds of the form $(CH_3)_3N^+(CH_2)_n\text{-}N^+(CH_3)_3$; phenyl-TMA, phenyltrimethylammonium iodide; methylfurmethide, 5-methylfurfuryltrimethylammonium iodide.

RESULTS

Effects of reduction on responsiveness to agonists. DTT was found to have fully reversible effects on the preparation when it was applied in concentrations between 0.1 and 1 mM for a few minutes at 37° (pH 7.4). As is described in more detail below, the muscle remained in a more or less stable condition after it had been exposed to DTT, until the effect was reversed by DTNB. The concentration of DTNB required to do this was about 0.5 mM. In most experiments 1 mM DTNB was applied for 10 min, and this completely restored the tissue to its original responsiveness. In some experiments the effect of reduction was observed by keeping 0.1 mM DTT in the organ bath while the test responses were being elicited; in others, particularly when alkylating agents, which might have reacted with DTT in solution, were being studied, 1 mM DTT was applied for 10 min and then washed out before the drug test was carried out. In preliminary tests it was found that the effect of 0.1 mM DTT took 15–30 min to develop fully, whereas 1 mM DTT acted in less than 5 min, the final effect being the same at these two concentrations.

Figure 1 shows that the same kind of reversal of the actions of carbachol and

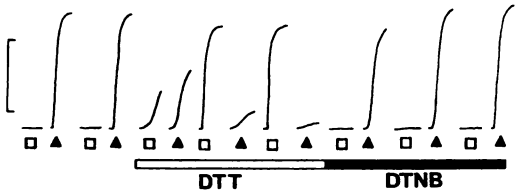

FIG. 1. *Effect of DTT (0.1 mM) on responses to carbachol and hexamethonium.*

The tracing is of isometric contractions of a strip of chick biventer muscle. Carbachol, 12 μM (▲), and hexamethonium, 0.1 mM (□), were applied alternately throughout the experiment, for 1 min each at 4-min intervals. The recording was stopped between drug applications. The DTT was washed out after the final carbachol application indicated. DTNB (0.4 mM) was then applied 30 sec before the next application of hexamethonium. The calibration represents 300 mg.

hexamethonium that Karlin and Winnik (4) found on the electroplax also occurs in chick muscle. In the control period, carbachol (12 μM) produced a response that was about 70% of maximal, whereas alternating doses of hexamethonium (0.1 mM) had no stimulant effect. In the presence of 0.1 mM DTT the effect of carbachol gradually disappeared, while at the same time a stimulant action of hexamethonium developed. DTNB (0.4 mM) rapidly abolished the action of hexamethonium and more gradually restored the sensitivity of the muscle to carbachol. After the DTNB was washed out the muscle usually gave a slow contraction, which took 10–15 min to subside. The contraction was not affected by tubocurarine, and only occurred if the preparation had previously been treated with DTT. Measurements of drug effects were made only after this contraction had disappeared. The cycle of reduction and reversal could be repeated several times without apparent ill effect on the muscle.

Hexamethonium caused no contraction in normal preparations, even in concentrations exceeding 1 mM. Indeed, it was found to act as a conventional competitive antagonist of rather low potency to carbachol, the equilibrium constant estimated by dose ratio measurements (12) being about 0.15 mM. This implies that hexamethonium had a measurable affinity for receptors in the normal preparation, so that the appearance of stimulant activity after the application of DTT could not have been due only to an increase in the affinity of hexamethonium for the receptors; it is necessary to postulate that the consequences of the occupation of the receptors by hexamethonium were also modified, in the sense that hexamethonium became an agonist rather than a pure antagonist.

Contractions elicited by KCl (which depolarizes the membrane directly without involving receptors) and by caffeine (which is thought to act by releasing calcium from the sarcoplasmic reticulum) were unaltered by DTT, making it unlikely that DTT had affected the muscle nonspecifically.

The action of DTT was not affected if it was applied in the presence of 30 μM tubocurarine, a concentration sufficient to block 98% of the receptors. Thus, occupation by tubocurarine did not appear to protect the receptors against reduction by DTT. In order to test whether the same receptors were involved in the stimulation by hexamethonium in the reduced preparation and by carbachol in the normal preparation, the receptors were blocked irreversibly in the normal preparation with 5 μM $DNC_{10}M$ for 10 min, causing an occupancy of about 0.95. The muscle was then exposed to DTT, and it was found that it did not develop the usual sensitivity to hexamethonium. We cannot say whether this was due to the alkylated receptors being protected against reduction, or (which is more likely) to the alkylation of the site with which hexamethonium combines, but in either case the implication is that the same receptors are involved in the stimulant actions of carbachol and hexamethonium.

In studying quantitatively the effects of DTT treatment on different agonists, the most reproducible results were obtained when the DTT was applied at 1 mM for 10 min and washed out before the tests were made. Test doses of the agonist were given between 5 and 20 min after washing out the DTT. This constant time sequence was used to avoid variability due to the slow decline in sensitivity (see Fig. 5 for an extreme example), which sometimes occurred after the muscle had been exposed to DTT. Figure 2 shows some examples of the way in which the sensitivity to different agonists was changed by DTT. Except in the case of hexamethonium, the slope of the log dose-response curves for different agonists was unchanged by treatment with DTT, although the position of the curve (reflecting the potency of the drug) was altered. This enabled the effect of DTT treatment to be expressed numerically as the ratio by which the concentration of a drug producing a given response was altered. We made measurements with 27 different agonists, each on three muscles, as shown in Table 1, including the two homologous series of mono- and bistrimethylammonium compounds. Where the activity ratio given is less than 1.0, the compound became less active after treat-

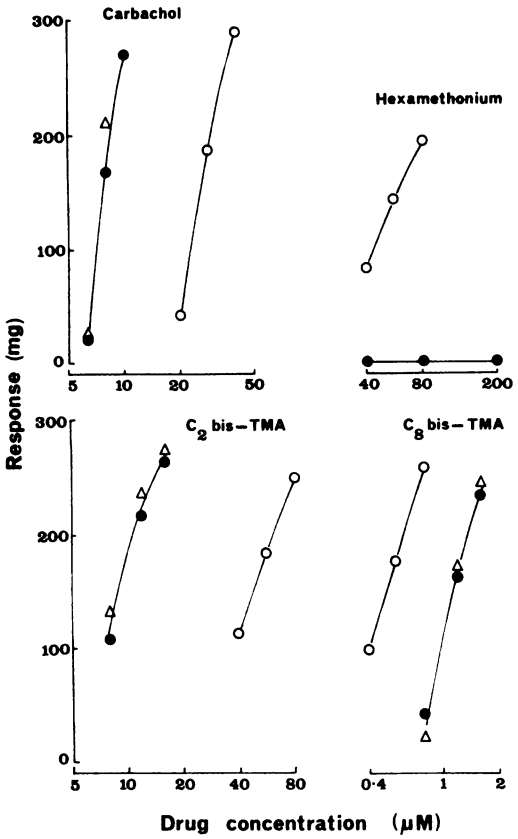

FIG. 2. *Effect of DTT on agonist log dose-response curves*

Each panel gives the result of a separate experiment. In each experiment a control dose-response curve (●) was obtained. DTT (1 mM) was then applied for 10 min, and the agonist was tested again (○). DTNB (1 mM) was then applied for 10 min, and more responses were recorded (△). Except with hexamethonium, the experimental and control curves were approximately parallel. Two of the drugs illustrated (hexamethonium and C_8-bis-TMA) became more active after treatment with DTT, while the other two (carbachol and C_2-bis-TMA) became less active. After DTNB treatment their activities returned to the control values.

ment with DTT; a ratio greater than 1.0 means that the compound became more active. The activity ratio is plotted as a function of chain length for the two homologous series in Fig. 3. In general, the monoquaternary series became less active after DTT, the largest effect being with the C_4, C_5, and C_6 compounds. The activities of the other compounds, except for tetramethylammonium, were little affected.

In tests with the bisquaternary series, the C_3, C_4, and C_5 compounds showed no stimulant activity either before or after DTT treatment. C_2-bis-TMA was greatly reduced in activity, whereas C_6-bis-TMA, as already described, was converted from an antagonist to an agonist (the potency ratio is therefore not calculable). C_7- and C_8-bis-TMA were potentiated, while longer-chain compounds were more or less unaffected in potency. The results obtained with other monoquaternary agonists are also shown in Table 1. Three of these (neostigmine, edrophoninum, and *m*-hydroxyphenyl-TMA) are better known as anticholinesterases than as cholinergic agonists, but all three cause contraction of the chick biventer cervicis. This contraction is inhibited by tubocurarine but is scarcely affected by eserine (which itself causes no contraction). It is therefore unlikely to be the result of enhancement of the action of endogenously released acetylcholine by inhibition of cholinesterase, and is much more likely to be a direct postsynaptic action of these compounds. Of these miscellaneous agonists, only neostigmine became substantially more active after treatment with DTT. The rest were either unchanged (methylfurmethide) or reduced in potency.

Because of the rather selective effect of DTT in increasing or reducing the potency of individual compounds, it was of interest to see how the over-all structure-activity relationships within the two homologous series were modified by DTT. For each compound, the concentration (MC_{50}) giving 50 % maximal contraction of normal preparations was determined (Table 1), the maximal contraction being obtained with a high concentration of carbachol (40 μM). The potency was expressed as the reciprocal of MC_{50}. The potency of the compound toward the DTT-treated muscle was obtained by multiplying the potency toward the untreated preparation by the potency ratio obtained with DTT. Figure 4 shows the relation between potency and chain length for control and DTT-treated muscles. In neither series was the over-all shape

TABLE 1

Effect of DTT on stimulant potency of various drugs on chick muscle

MC_{50} is the molar concentration required to give a contraction of a normal muscle 50% of the size of the maximum obtainable with carbachol. The activity ratio is the reciprocal of the ratio by which the equiactive concentration changed after the muscle had been treated with 1 mM DTT for 10 min. The means and standard errors of measurements made on three muscles are given.

Agonist	MC_{50}	Activity ratio
Carbachol	$7.98\ (\pm 0.69) \times 10^{-6}$	0.21 ± 0.04
Acetylcholine[a]	$2.53\ (\pm 0.24) \times 10^{-7}$	0.18 ± 0.02
Suxamethonium	$2.13\ (\pm 0.15) \times 10^{-7}$	0.46 ± 0.03
Nicotine	$3.13\ (\pm 0.79) \times 10^{-6}$	0.24 ± 0.02
Methylfurmethide	$3.39\ (\pm 0.31) \times 10^{-5}$	1.14 ± 0.07
Phenyl-TMA	$9.77\ (\pm 0.11) \times 10^{-6}$	0.37 ± 0.04
m-Hydroxyphenyl-TMA	$2.46\ (\pm 0.26) \times 10^{-5}$	0.43 ± 0.05
Edrophonium	$4.40\ (\pm 0.61) \times 10^{-5}$	0.43 ± 0.02
Neostigmine	$2.45\ (\pm 0.53) \times 10^{-5}$	2.60 ± 0.54
C_n-TMA compounds		
$n = 1$	$6.24\ (\pm 0.55) \times 10^{-5}$	0.56 ± 0.04
$n = 2$	$1.35\ (\pm 0.09) \times 10^{-4}$	0.83 ± 0.08
$n = 3$	$1.77\ (\pm 0.11) \times 10^{-4}$	0.79 ± 0.06
$n = 4$	$2.75\ (\pm 0.03) \times 10^{-6}$	0.24 ± 0.01
$n = 5$	$1.68\ (\pm 0.07) \times 10^{-6}$	0.31 ± 0.01
$n = 6$	$2.13\ (\pm 0.08) \times 10^{-6}$	0.40 ± 0.03
$n = 7$	$3.04\ (\pm 0.20) \times 10^{-6}$	0.96 ± 0.02
$n = 8$	$5.40\ (\pm 0.58) \times 10^{-6}$	1.17 ± 0.17
C_n-bis-TMA compounds:		
$n = 2$	$8.33\ (\pm 0.33) \times 10^{-6}$	0.17 ± 0.01
$n = 7$	$1.83\ (\pm 0.28) \times 10^{-5}$	9.30 ± 0.41
$n = 8$	$9.60\ (\pm 0.40) \times 10^{-7}$	2.15 ± 0.13
$n = 9$	$2.03\ (\pm 0.13) \times 10^{-7}$	1.01 ± 0.09
$n = 10$	$1.21\ (\pm 0.02) \times 10^{-7}$	0.83 ± 0.03
$n = 11$	$8.40\ (\pm 0.00) \times 10^{-8}$	0.87 ± 0.08
$n = 12$	$6.32\ (\pm 0.64) \times 10^{-8}$	0.98 ± 0.02
$n = 13$	$5.45\ (\pm 0.63) \times 10^{-8}$	0.95 ± 0.14
$n = 14$	$5.55\ (\pm 0.28) \times 10^{-8}$	1.30 ± 0.07
$n = 15$	$8.32\ (\pm 0.67) \times 10^{-8}$	$0.97 - 0.08$

[a] Measured in the presence of 10 μM physostigmine.

of the relationship between potency and chain length grossly altered by treatment with DTT.

Stability of DTT-treated preparation. The DTT-treated preparation did not usually produce consistent drug responses for a long time, although the muscle could normally be restored to its original responsiveness by reoxidation with DTNB. When DTT was applied for a few minutes and then washed out, the effect sometimes subsided, presumably because of spontaneous re-formation of the disulfide bridges. More often, however, a progressive decline in sensitivity to all agonists occurred, and this effect could be so marked as to prevent any quantitative measurements. An example is shown in Fig. 5. The agonist was C_8-bis-TMA, at a concentration which gave only a very small response during the control period. After DTT treatment the contraction was initially increased, but this was not sustained. This was not due to spontaneous recovery from the action of DTT, because the effects of other agonists, such as carbachol, which were made less active by DTT, showed a parallel decline. Moreover, a second application of DTT did not restore the responsiveness to drugs such as C_8-bis-TMA. DTNB could, however, restore normal

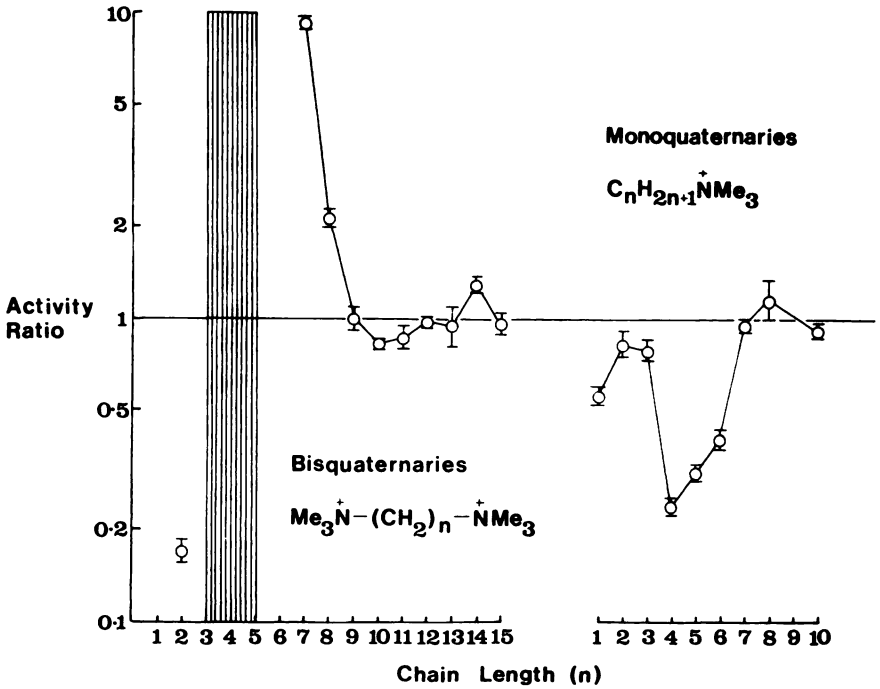

FIG. 3. *Change in potency of agonists caused by DTT*

The figure shows logarithmic plots of the activity ratios resulting from treatment with DTT (1 mM for 10 min), for two homologous series of agonists. The hatched area in the plot for the bisquaternary compounds shows those compounds (C_3-bis-TMA, C_4-bis-TMA, and C_5-bis-TMA) that were inactive both before and after application of DTT. Each point is the mean (± standard error) of three observations.

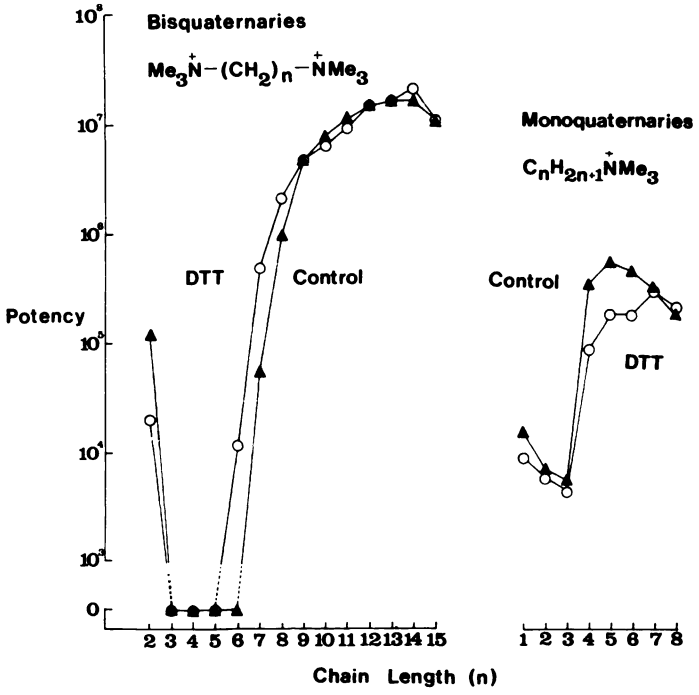

FIG. 4. *Effect of DTT on potency of different agonists*

The two panels refer to the same groups of drugs as shown in Fig. 3. The measure of potency used is the reciprocal of the molar concentration needed to give a half-maximal contraction. ▲, control preparations; O, DTT-treated preparations.

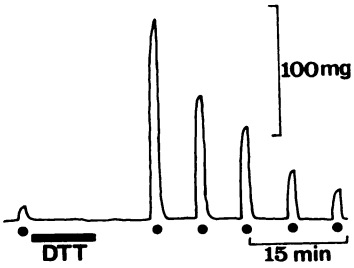

FIG. 5. *Decline in sensitivity occasionally seen after DTT*

The tracing shows contractions in response to 0.8 μM C_8-bis-TMA, (●), in which the decline in sensitivity following DTT (1 mM for 10 min) was unusually great.

sensitivity to the muscle even after it had undergone this secondary decline in the presence of DTT. This effect, also described for the action of decamethonium on the eel electroplax by Podleski, Meunier, and Changeux (13), was only occasionally as pronounced as it is in Fig. 5. In most experiments the tendency was present, but was not large enough to interfere seriously with measurements made within about 20 min of washing out the DTT.

Interaction of DTT with antagonists. In four preparations the potency of tubocurarine as an antagonist was measured in the presence of 0.1 mM DTT. The dose ratio method was used as described previously (11), and experiments were made with four different agonists—carbachol, methylfurmethide, C_7-bis-TMA, and decamethonium—some of which became more and some less active in the presence of DTT (Table 1). In these experiments 0.1 mM DTT was allowed to act for 15–30 min; test responses to the agonist in question were then elicited (in the presence of DTT) in order to obtain a preantagonist log dose-response curve. The antagonist was applied, still in the presence of DTT, and a further set of test responses was obtained. The antagonist was then washed out and a check was made to see that the sensitivity had returned more or less to its preantagonist level. In most experiments the recovery was satisfactory; occasionally this was not so, possibly because of a progressive decline in sensitivity in the presence of DTT (see earlier section). Such experiments were rejected.

Tubocurarine caused the usual shift to the right of the agonist log dose-response curve without causing any change in slope. The results are plotted as (dose ratio − 1) with respect to tubocurarine concentration (12) in Fig. 6. The linearity of these plots shows that tubocurarine antagonism obeys the competitive equation (12): the equilibrium constant is given by the reciprocal of the slope of the line. The equilibrium constant was calculated for each point and the results were averaged, giving a value of $3.78\ (\pm 0.08) \times 10^{-7}$ M ($n = 19$) in normal solution, and $1.59\ (\pm 0.07) \times 10^{-7}$ M ($n = 10$) in the presence of DTT. Thus DTT increased the affinity of tubocurarine for the receptors by a factor of 2.4. There was no evidence that tubocurarine discriminated between one agonist and another either before or after DTT treatment.

Interaction with irreversible antagonists. Karlin (6) has shown that DTT treatment of the electroplax greatly facilitates receptor alkylation by certain quaternary maleimide derivatives. On the other hand, Podleski and his co-workers (13) found that the irreversible blocking action of *p*-(trimethylammonium) benzenediazonium fluoroborate is lost after treatment of the electroplax with DTT, a reversible depolarizing effect appearing in its place. It was thus of interest to study the effect of DTT treatment on the action of receptor-alkylating agents on chick muscle. The alkylating agents tested were diphenyldecamethonium mustard ($DPC_{10}M$) and dinaphthyldecamethonium mustard ($DNC_{10}M$), whose properties we have reported on previously (11, 14).

The experiments were done by measuring the carbachol dose ratio produced by a given concentration of $DPC_{10}M$ or $DNC_{10}M$ applied for a given time, and comparing this with the dose ratio obtained when the muscle was exposed to DTT (1 mM for 10 min) before the blocking agent was applied, and then to DTNB (1 mM for 10 min) before the test doses of carbachol were given. Thus the carbachol dose ratio was measured on the muscle in an unreduced state.

Typical results are shown in Fig. 7. With

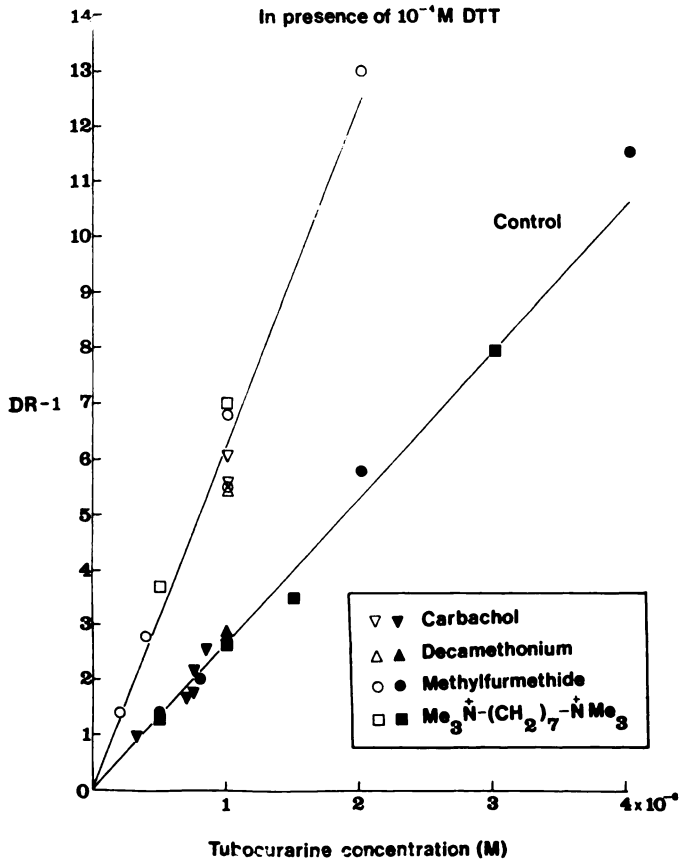

FIG. 6. *Effect of DTT on potency of tubocurarine*

Various agonists were used as indicated. Measurements of the dose ratios produced by several concentrations of tubocurarine were made, and (dose ratio −1) was plotted against tubocurarine concentration. The solid symbols represent control measurements made in the absence of DTT. The open symbols represent measurements made in the presence of 0.1 mM DTT. In each case the plot was linear, as the competitive model demands. However, the line was steeper when the measurements were made in the presence of DTT, showing that tubocurarine was more potent in this situation.

both $DPC_{10}M$ and $DNC_{10}M$ the blocking action was increased when the drugs were applied after DTT treatment. With $DNC_{10}M$ the effect was not very great. In Fig. 7, $DNC_{10}M$ (0.8 μM for 2 min) applied to the normal muscle produced a dose ratio of 1.2 (17% of the receptors alkylated), whereas the same concentration of $DNC_{10}M$ applied after DTT produced a dose ratio of 1.5 (33% of the receptors alkylated). Other results are given in Table 2, and show a definite but small effect. With $DPC_{10}M$ the antagonism was very greatly increased by treatment with DTT. Thus, in the experiment shown in Fig. 7, $DPC_{10}M$ (0.6 μM for 10 min) caused virtually no blockade in a normal muscle, but gave a dose ratio of 5.0 (80% of the receptors blocked) when applied after DTT. To produce 80% blockade with $DPC_{10}M$ in a normal muscle in 10 min requires at least 10 times the concentration used in the experiment of Fig. 7. Table 2 collects the results of a number of experiments showing the increased effectiveness of $DPC_{10}M$ following treatment of chick muscle with DTT. The effect is independent of the agonist used to test the blockade.

Since irreversible blockade of the receptors must be assumed to occur in two stages, reversible association followed by alkylation,

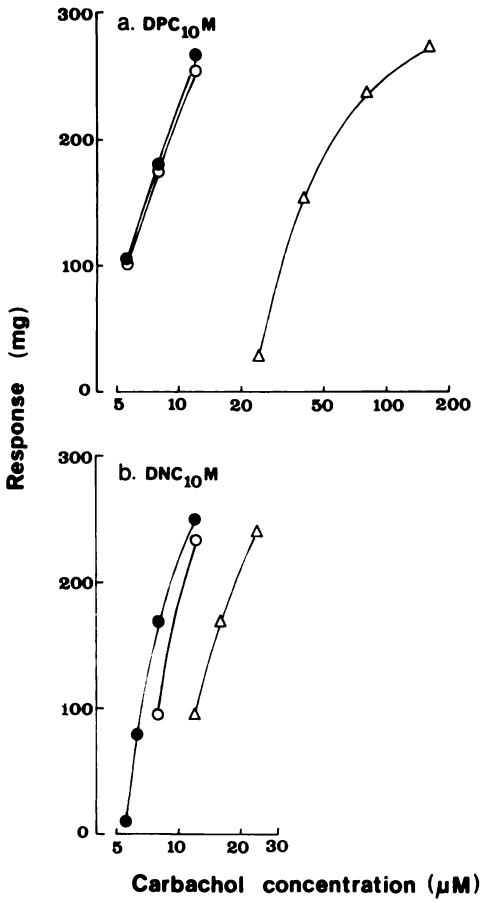

FIG. 7. *Effect of DTT on potency of irreversible antagonists*

The figure shows log dose-response curves to carbachol in two preparations (a and b). In each experiment a control dose-response curve was obobtained (●), then the antagonist was applied ($DPC_{10}M$, 0.6 μM for 10 min; $DNC_{10}M$, 0.8 μM for 2 min). These applications caused little blockade (○). DTT (1 mM for 10 min) was then applied, followed by the same application of antagonist as had been used before. DTNB (1 mM for 10 min) was then applied, and a final dose-response curve (△) was determined. In both cases a larger blockade was produced than had occurred when the antagonist was applied to the untreated muscle, but the enhancement was much greater with $DPC_{10}M$ than with $DNC_{10}M$.

we wished to find which of these steps was facilitated by DTT treatment. A few experiments were therefore conducted with DPC_{10}, the nonalkylating analogue of $DPC_{10}M$. The existence of the metaphilic effect (11) with DPC_{10} made it difficult to measure an equilibrium constant by the usual method, but, provided that the dose ratio was small, the degree of antagonism produced by a given concentration could be measured and compared with similar results obtained after DTT treatment. In two experiments it was found that DTT treatment roughly doubled the blocking potency of DPC_{10}, an effect similar to that seen with tubocurarine. Since the enhancement seen with $DPC_{10}M$ was more than 10-fold, it is likely that it represented mainly an increase in the rate of alkylation, although an increase in reversible binding may have made some contribution.

DISCUSSION

Our results on chick muscle closely resemble those obtained on the electroplax (3–7, 13). Although Karlin used a different method of expressing the change in activity, his results with acetylcholine, carbachol, TMA, C_4-TMA, and hexamethonium agree approximately with ours. With decamethonium, Karlin (6) and Podleski *et al.* (13) found an increase in activity after DTT treatment, whereas we observed a very small decrease.

Our findings with the alkylating agents $DPC_{10}M$ and $DNC_{10}M$ are similar to Karlin's (6) observations on quaternary maleimide compounds, which become potent, irreversible blocking agents after DTT treatment. On the other hand, Podleski *et al.* (13) obtained the opposite result with *p*-(trimethylammonium) benzenediazonium fluoroborate, which acts as an irreversible blocking agent normally, but as a reversible depolarizing drug after DTT.

We had hoped that a fairly systematic study of structure-activity relationships in normal and DTT-treated tissue might bring to light generalizations from which the type of conformational change caused by DTT might be deduced, but it is extremely difficult to perceive any clear pattern in the results. Among the monoquaternaries, it is true that three similar compounds, edrophonium, *m*-hydroxyphenyl-TMA, and phenyl-TMA, were about equally affected by DTT, but a similar compound, neostigmine, was affected in the opposite direction.

TABLE 2

Effect of DTT on blocking action of DPC_{10} M and DNC_{10} M in chick muscle

r_1 is the agonist dose ratio produced by the antagonist applied to a normal preparation. r_2 is the dose ratio after application of 1 mM DTT for 10 min, leaving the preparation for 15 min, and then applying 1 mM DTNB for 10 min. r_3 is the dose ratio after application of 1 mM DTT for 10 min, then the antagonist, and finally 1 mM DTNB for 10 min. Comparison of r_3 and r_1 thus shows the effect of reduction on the block produced by the antagonist.

Expt	Antagonist	Concentration	Time of application	Agonist	r_1	r_2	r_3
		μM	min				
1	DPC_{10} M	2.4	15	Carbachol	1.25	1.3	9.0
2	DPC_{10} M	0.6	15	Carbachol			10.0[a]
3	DPC_{10} M	0.6	10	Carbachol	1.0	1.1	5.0
4	DPC_{10} M	0.6	10	Methylfurmethide			6.5
5	DPC_{10} M	0.6	10	Carbachol			3.4
				C_7-bis-TMA			3.9[a]
6	DPC_{10} M	0.6	10	Carbachol	1.0		10.0
7	DPC_{10} M	0.6	15	Carbachol			4.4
				C_7-bis-TMA			5.4[a]
8	DPC_{10} M	0.15	10	Carbachol			1.8
				C_8-bis-TMA			2.3
9	DPC_{10} M	0.6	10	Carbachol			3.8
				C_8-bis-TMA			4.4
10	DNC_{10} M	0.8	2	Carbachol	1.2	1.0	1.8
11	DNC_{10} M	0.8	2	Carbachol	1.2	1.0	1.5
12	DNC_{10} M	0.4	2	Carbachol	0.9		1.4

[a] Curves flattened; r measured at lower end of curve.

When the effects of DTT treatment on potency within the two homologous series are shown as in Fig. 4, it is seen that DTT has a relatively small effect on the over-all pattern. Within the bisquaternary series, the main effect is to reduce activity roughly to that of the homologue with one fewer methylene group, although not with compounds longer than C_8-bis-TMA.

In leech muscle (11, 15) and in the electroplax (13) there is evidence that two types of cholinergic receptor exist: in both these tissues carbachol appears to act at different receptors from decamethonium. The opposite effects of DTT on the depolarizing activity of different agonists might thus be explained quite simply if disulfide bond reduction facilitated the activation of one type of receptor but inhibited activation of the other. In chick muscle, however, there is evidence against the existence of more than one type of cholinergic receptor. Thus tubocurarine blocks equally the effects of carbachol, suxamethonium, methylfurmethide, C_8-bis-TMA, and decamethoniom; gallamine also fails to discriminate in its blocking action between carbachol and suxamethonium (11). Moreover, desensitization of carbachol receptors in chick muscle can be brought about by many different cholinergic agonists, and shows no selectivity (16); similarly, the metaphilic effect (11), which appears to be due to the same process as desensitization (14), also fails to discriminate among different agonists. In leech muscle, where the selective blocking action of tubocurarine and other antagonists suggests that more than one type of receptor exists (11, 15), desensitization and the metaphilic effect show corresponding selectivity.

The results obtained with tubocurarine also make it unlikely that an appreciable fraction of the receptors exists in the reduced state in a normal preparation, or that DTT used under these conditions leaves an appreciable proportion of receptors in the

unreduced state. Since the dissociation constant for tubocurarine was reduced by a factor of 2.4 by DTT treatment, incomplete reduction by DTT would result in two populations of receptors with differing affinities for tubocurarine and with differing susceptibilities to the various agonists. This heterogeneity would mean that the dose ratio plot (Fig. 6) would be nonlinear, and also that the graphs obtained with different agonists would not be superimposable. Since the graphs were linear, and results with different agonists agreed closely, we conclude that chick muscle possesses a single, homogeneous population of receptors, which was fully reduced by DTT in these experiments.

In some of our preparations, the responses declined progressively with time after the muscle had been treated with DTT. A similar effect has been described for the electroplax (13), although in that tissue the effect of decamethonium decreased while that of carbachol did not. In our experiments, the decline affected all agonists equally. It was a highly variable effect, and therefore difficult to study systematically, and we have no evidence about the mechanism involved.

Irreversible block of the receptors can be assumed to occur in two stages:

$$X + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} XR \text{ (reversible complex)}$$

$$\xrightarrow{k_3} X\text{-}R \text{ (alkylated complex)}$$

The blocking action of $DPC_{10}M$ was increased at least 10-fold when it was tested on a DTT-treated muscle, whereas the affinity of the reversible analogue, DPC_{10}, was no more than doubled. This makes it likely that the main effect of DTT was to increase the rate constant, k_3, for the alkylation step, although an increase in the affinity constant, k_1/k_2, may have contributed.

The much smaller effect of DTT on the blocking action of $DNC_{10}M$ is compatible with this mechanism if, in normal tissue, the ratio k_3/k_2 is large for $DNC_{10}M$ (so that a receptor reversibly occupied by $DNC_{10}M$ is much more likely to be alkylated than vacated). An increase in k_3 produced by DTT would then have relatively little effect on the rate at which alkylation proceeds. With $DPC_{10}M$ the ratio k_3/k_2 may normally be small, so that an increase in k_3 would result in a large increase in the blocking potency. The present evidence does not indicate whether the increase in k_3 is due to greater accessibility of the group or groups that are alkylated in the normal, unreduced receptor, or to the alkylation of the reduced sulfhydryl groups that normally form a disulfide bridge.

The increase in the blocking action of $DPC_{10}M$ caused by DTT called to mind the metaphilic effect (11, 14, 16) whereby desensitization of the tissue by agonists increases the blocking action of $DPC_{10}M$ and $DNC_{10}M$, and we considered whether the action of DTT might be equivalent to the process of desensitization. Had this been so, however, desensitization should have (like DTT) *increased* the potency of certain agonists, such as C_7-bis-TMA, whereas we found that a desensitizing dose of carbachol diminished the response to C_7-bis-TMA as much as to other agonists. Furthermore, although the metaphilic effect was more pronounced with $DNC_{10}M$ than with $DPC_{10}M$, the increase in potency after DTT was greater with $DPC_{10}M$. Thus it seems unlikely that desensitization and the metaphilic effect were both the result of disulfide bond reduction.

Our findings are consistent with the conclusion of Karlin and Winnik (4) that the cholinergic receptor is a protein containing a disulfide bond reduced by dithiothreitol. After such reduction the specificity of the receptor for different agonists and antagonists is modified, without the receptors losing their functional properties.

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