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EFFECTS OF DITHIOTHREITOL ON END-PLATE CURRENTS

BY D. A. TERRAR

From the Department of Pharmacology and Therapeutics, The Middlesex Hospital Medical School, London W1P 7PN

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SUMMARY

1. End-plate currents have been studied in frog *cutaneus pectoris* nerve–muscle preparations mounted in continuously flowing solution, using the voltage clamp technique.

2. Exposure of the muscle to 1 mM-dithiothreitol reduced the amplitude of end-plate currents by a factor of 2·7 (mean; range 1·6–3·4; twelve fibres).

3. 1 mM-dithiothreitol also caused a 2·7-fold (2·3–3·1) increase in the rate of decay, and a 1·4-fold (1·3–1·6) decrease in the time to peak of end-plate currents. During the onset of action of dithiothreitol, there was little or no indication of departure of end-plate current decay from a simple exponential.

4. Dithiothreitol actions on amplitude and decay of end-plate currents developed with similar time courses and both effects were slower in onset at pH 7·2 than at pH 8·5.

5. The actions of dithiothreitol were reversed by exposure of the muscle to 1 mM-5,5′-dithio-bis-(2-nitrobenzoic acid).

6. Following dithiothreitol treatment, the rates of decay of end-plate currents continued to depend on membrane potential; there was little or no change in the slope of the relation between ln (rate of decay) and membrane potential, consistent with little or no change in the dipole moment of a gating molecule for ion channels.

7. Dithiothreitol changed the relation between peak end-plate current and membrane potential, so that peak conductance increased at more negative membrane potentials; this finding could be accounted for in terms of the closure of ion-channel gates becoming faster though remaining voltage-sensitive after exposure to dithiothreitol.

8. It is concluded that dithiothreitol causes changes in the kinetics of gating of ion channels associated with receptors and that these changes accompany changes in the binding of ACh to receptors.

INTRODUCTION

Dithiothreitol, which reduces disulphide bonds in proteins (Cleland, 1964), has been used in the isolation of nicotinic acetylcholine receptors from *Electrophorus* electroplaques (Karlin, Cowburn & Reiter, 1973), where it is thought to reduce disulphide bonds located approximately 1 nm from an anionic site on the receptor (Karlin, 1969). Responses to cholinergic agonists in this and other tissues containing nicotinic acetylcholine receptors have been shown to be modified by dithiothreitol

(Karlin & Bartels, 1966; Karlin & Winnik, 1968; Albuquerque, Sokoll, Sonesson & Thesleff, 1968; Mittag & Tormay, 1970; Rang & Ritter, 1971; Fleisch, Krzan & Titas, 1974; Brown & Kwiatkowski, 1976). In frog skeletal muscle this compound reduces the amplitude of end-plate potentials (Ben-Haim, Landau & Silman, 1973; Landau & Ben-Haim, 1974) and an analysis of acetylcholine (ACh) 'noise' in the same preparation suggested that such an effect results from a reduction in conductance and lifetime of ion channels opened by ACh (Landau & Ben-Haim, 1974; Ben-Haim, Dreyer & Peper, 1975). The present study examines the effect of dithiothreitol on end-plate currents in frog skeletal muscle under voltage-clamp conditions in an attempt to relate effects on receptors with effects on the gating of associated ion channels. Some preliminary results have been reported previously (Terrar, 1976).

METHODS

The experiments were done throughout the year on nerve cutaneus pectoris preparations of the frog *Rana temporaria*. The muscle was pinned on Sylgard resin in a channel (10 mm wide and 4 mm deep) milled from a perspex block. A continuous stream of solution flowed at 14 ml. min^{-1} through the channel, bathing the muscle, from a reservoir mounted above the bath; the composition of this solution could be changed rapidly by means of a tap connected to the inflow of the bath by a short length of narrow, silicone-rubber tubing (Jenkinson & Terrar, 1973). Reservoirs containing bathing solutions were surrounded by water jackets maintained at 20 °C, and in later experiments a thermistor mounted in the solution next to the muscle was used to check that the temperature remained within 1° of 20 °C.

The standard bathing solution contained (mM): NaCl, 116; KCl, 2·0; $CaCl_2$, 1·8; Na_2HPO_4, 1·92; NaH_2PO_4, 0·48. The pH of this solution was 7·2. A solution of pH 8·5, which was necessary for some experiments, was similar to the standard solution but contained 5·0 mM-Tris base (Sigma) in place of the sodium phosphates, the pH being adjusted with HCl.

To prevent contraction when the nerve was stimulated the muscles were pre-treated with hypertonic glycerol (Gage & Eisenberg, 1967; Howell, 1969). The preparation was first immersed for 60 min in standard bathing solution to which 500–800 mM-glycerol had been added, and then transferred to the standard solution for 30–60 min before use. Resting potentials of fibres selected for study were more negative than −35 mV, often close to −50 mV which was the standard potential at which it was decided to 'voltage clamp' every fibre except in those experiments where the membrane potential was to be varied.

The voltage-clamping apparatus used was similar to that described previously (Connor & Stevens, 1971; Magleby & Terrar, 1975). Under voltage clamp conditions, the maximum membrane potential variations at the site of the voltage electrode were less than 2–5% of the difference between the equilibrium potential and the holding potentials. Voltage recording micro-electrodes were filled with 3 M-KCl. Current micro-electrodes were filled with 3 M-KCl or 2–4 M-$KMeSO_4$ (Jenkinson & Terrar, 1973); no differences were observed between them. Micro-electrode resistances were 2–8 MΩ and the 10–90% rise time of the voltage response of these micro-electrodes to a 1 nA square wave of current applied in the bathing solution was less than 120 μsec. Voltage and current data were displayed on Tektonix 565 and 564B oscilloscopes and recorded on a Racal Store 4 FM tape recorder at 15 in. sec^{-1} (1 dB down at 5 kHz). Time constants characterizing the decay of end-plate currents were determined either from semilogarithmic plots against time of the currents measured by hand, or from the slopes of lines generated by a logarithmic amplifier (Analog Devices 755P, or Ancom 15 LN-1; see Magleby & Terrar, 1975). The DC level of the signal fed into the logarithmic amplifier was continuously monitored to prevent errors resulting from possible base line shifts. Similar results were obtained from hand measured data and from data processed by the logarithmic amplifier.

End-plates were localized by the presence of fine nerve terminals. The voltage and current electrodes were then placed within 50–100 μm of each other at that region of the muscle fibre which gave the maximum amplitude of end-plate potential or current, as determined by moving the electrodes along the fibre. Unless otherwise stated, electrodes were kept in place during

solution changes. Data were rejected if drift in the recording electrode exceeded 3 mV. The nerve was stimulated at 0·5 pulses sec^{-1}.

Dithiothreitol and 5,5′-dithio-bis-(2-nitrobenzoic acid) were obtained from Sigma. Dithiothreitol was made up as a 1 mM solution immediately before use.

The statistical methods used were *t* tests, Wilcoxon rank tests, and regression lines (discussed in Colquhoun, 1971).

RESULTS

Effects of dithiothreitol on amplitude and time course of end-plate currents

The effect of 1 mM-dithiothreitol on end-plate currents recorded at a standard potential of −50 mV and at 20 °C are illustrated in Fig. 1. The four superimposed recordings of Fig. 1*A* are end-plate currents from one fibre before and after increasing exposures to dithiothreitol at pH 7·2 applied in the solution flowing over the muscle.

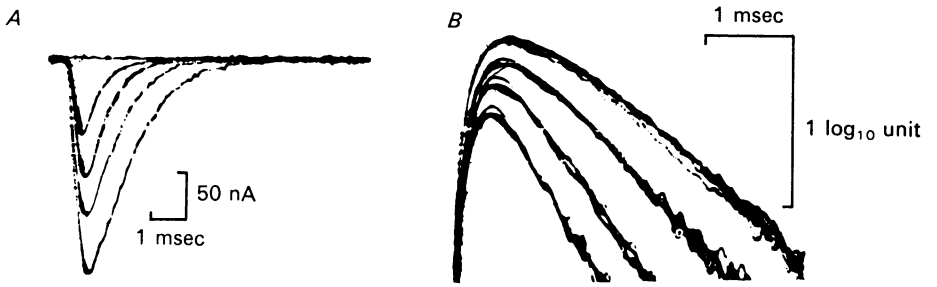

Fig. 1. Effect of 1 mM-dithiothreitol on end-plate currents. *A*, four superimposed end-plate currents recorded (in order of decreasing amplitude) after 0, 5, 10 and 20 min exposures to dithiothreitol. *B*, logarithmic transforms (generated by an operational amplifier, see Methods) of end-plate currents from same fibre, five superimposed currents for each of the four times of exposure to dithiothreitol shown in *A*. Fibre clamped at −50 mV.

It can be seen that dithiothreitol caused a progressive reduction in amplitude accompanied by an increase in the rate of decay of end-plate currents. The rate constant for decay (α, $msec^{-1}$) was measured either by plotting the end-plate currents semi-logarithmically against time or, as illustrated in Fig. 1*B*, from logarithmic transforms (generated by an operational amplifier) of the currents; both methods gave similar estimates for α determined from the approximately linear part of the curves, ignoring non-linear behaviour near the peak. At pH 7·2, the effects of dithiothreitol reached an approximately steady level in about 20–25 min. This might reflect conversion of all receptor molecules or associated ion channels to an altered molecular conformation in which the closing of gates for ion channels became more rapid. The possibility exists that before the effect of dithiothreitol reached a steady level there are two populations of receptors and associated ion channels, some with the characteristics of untreated muscle, and some with those of the fully reduced state. If this were so the end-plate currents should show two phases in their decay. It is clear from Fig. 1*A* and *B* (see also Terrar, 1976) that at an intermediate level of dithiothreitol action there was no obvious change from simple exponential decay. Observations of this kind were made consistently. The inability of the simple model of two populations of receptors and associated ion channels to account for these findings is further considered in the Discussion.

It was of interest to compare the time courses of the effects of dithiothreitol on amplitude and rate of decay of end-plate currents. These are shown in Fig. 2 both for the experiment on which Fig. 1 was based, (filled symbols) at pH 7·2, and for another fibre in a different muscle (open symbols) exposed to the same concentration of dithiothreitol at pH 8·5. It can be seen (1) that the actions of dithiothreitol both on amplitude and on rate of decay developed more rapidly at pH 8·5 than at pH 7·2 and (2) that at each pH the two effects developed with a similar time course. In

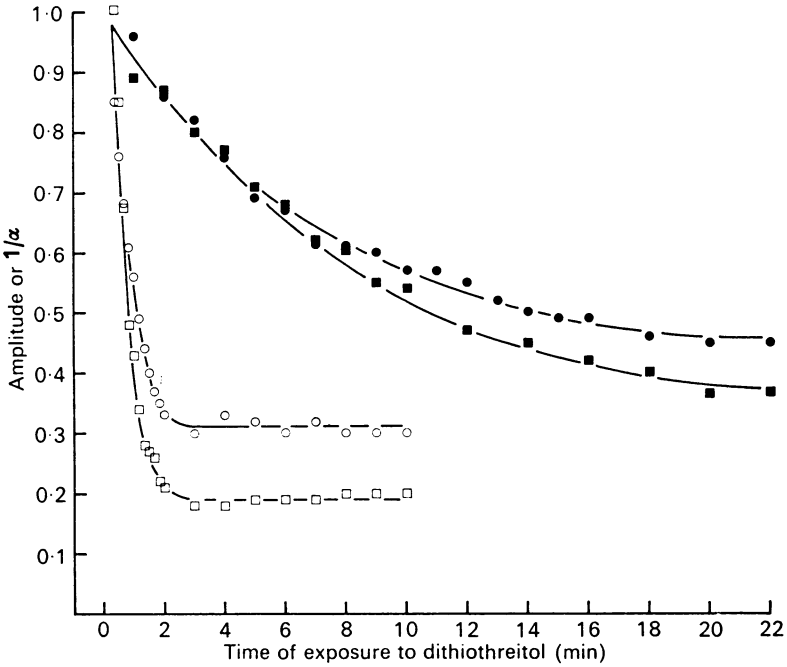

Fig. 2. Time course of the effects of 1 mM-dithiothreitol at pH 7·2 (filled symbols) and at pH 8·5 (open symbols). Squares represent amplitude and circles time constant of decay ($1/\alpha$), in each case expressed as a fraction of the value before exposure to dithiothreitol. Each point at pH 7·2 represents the mean of five or six end-plate currents; at pH 8·5, points represent individual currents up to 2 min, then means of two to six currents. Filled symbols from the fibre on which Fig. 1 was based.

six fibres at pH 7·2 the effects developed halfway towards an approximately steady level in about 5 min, whereas half-times for the effects in seven fibres at pH 8·5 were less than 2 min. Assuming pH does not influence the rate of access of dithiothreitol to its site of action, these different rates of action at the two pHs may reflect a pH-dependent reduction of disulphide bonds, though in making such a comparison it must be borne in mind that any delay in access is expected to contribute proportionately more to the half-time at pH 8·5 than at pH 7·2. An increased rate of reduction of disulphide bonds at high pH is expected in the light of Cleland's (1964) experiments where dithiothreitol was thought to have a more negative redox potential at pH 8·0 than at pH 7·0.

A more quantitative analysis of the rate of action of 1 mM-dithiothreitol at pH 7·2 was undertaken in four fibres. If both amplitude and $1/\alpha$ decay exponentially with

time after exposure to dithiothreitol, and with the same rate constant, then plots of the expressions $(a_t - a_\infty)/(a_0 - a_\infty)$ and $(1/\alpha_t - 1/\alpha_\infty)/(1/\alpha_0 - 1/\alpha_\infty)$ on a semilog scale against time should be linear and superimpose; in these expressions a_t and α_t are the amplitude and rate of decay at time t (min) exposure to dithiothreitol respectively, a_0 and α_0 at zero time, and a_∞ and α_∞ at infinite time (calculated from end-plate currents obtained after 20 min exposure assuming reactions to be 94% complete, as would be the case if the half-time were 5 min). Such a plot is shown in

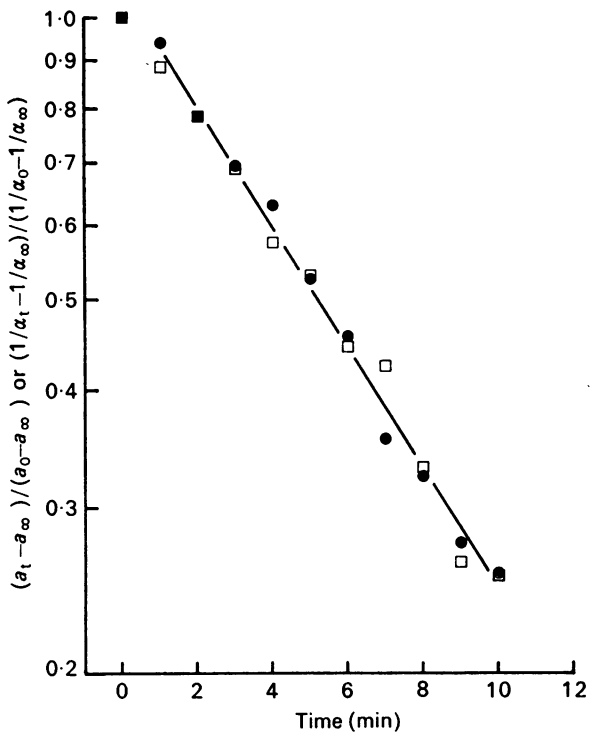

Fig. 3. Combined results from four fibres showing rate of onset of the action of 1 mM-dithiothreitol on amplitude (□) and rate of decay (●) of end-plate currents at pH 7·2, each point being the mean of measurements from all four fibres; see Results for explanation of ordinate. The regression line shown is fitted to all points between 1 and 10 min exposures to dithiothreitol and has a slope of 0·146 min^{-1}; slopes of regression lines fitted only to the data for amplitude or for rates of decay were 0·144 min^{-1} and 0·150 min^{-1}, respectively.

Fig. 3. It can be seen that the approach to a steady level of the actions of 1mM-dithiothreitol on amplitude and rate of decay of end-plate currents are approximately first order processes and that the rate constants for each process were similar. The 95% confidence limits for the rate constants were calculated as 0·130 min^{-1} and 0·157 min^{-1} for amplitude and 0·139 min^{-1} and 0·161 min^{-1} for decay.

Table 1 summarizes the effects of 1 mM-dithiothreitol once an approximately steady level was reached. Differences between the steady effects at the two pHs were small and it was concluded from two-sample t tests and Wilcoxon rank tests that none of these differences was significant at the 5% level. It can be seen that

there was a mean 2·7-fold reduction in amplitude (1·6–3·4), a 2·7-fold increase in the rate of decay (2·3–3·1), and a 1·4-fold decrease in the time to peak (1·3–1·6). It should be mentioned that even after treatment with dithiothreitol end-plate potentials still initiated action potentials in unclamped fibres with high (more negative than about −70 mV) resting potentials in glycerol-treated muscle, and that in normal, non-glycerol treated, muscle exposure to dithiothreitol as above at pH 7·2 or 8·5 did not prevent contraction in response to stimulation of the nerve; these findings presumably reflect the safety margin for neuromuscular transmission.

TABLE 1. Effect of 1 mM-dithiothreitol on the amplitude (a, nA), rate of decay (α, msec^{-1}) and time to peak (t, msec) of end-plate currents. Fibres one to six were exposed to dithiothreitol for 20–25 min at pH 7·2, fibres seven to twelve for 10 min at pH 8·5. All measurements were made at pH 7·2, 20 °C, −50 mV.

	Before dithiothreitol			After dithiothreitol					
Fibre	a_1	α_1	t_1	a_2	α_2	t_2	a_1/a_2	α_2/α_1	t_1/t_2
1	47	1·08	0·60	17	2·66	0·44	2·8	2·5	1·4
2	198	0·99	0·60	89	2·68	0·40	2·2	2·7	1·5
3	190	0·76	0·68	88	1·74	0·48	2·4	2·3	1·4
4	245	0·98	0·68	72	2·50	0·46	3·4	2·6	1·5
5	286	0·83	0·70	183	2·22	0·56	1·6	2·7	1·3
6	252	0·89	0·70	88	2·08	0·40	2·9	2·4	1·3
7	211	0·83	0·76	85	2·40	0·60	2·5	2·9	1·3
8	160	0·84	0·64	60	2·34	0·42	2·7	2·8	1·5
9	89	0·90	0·60	30	2·12	0·44	3·0	2·4	1·4
10	160	1·08	0·62	59	2·60	0·40	2·7	2·4	1·6
11	344	1·11	0·62	114	3·32	0·40	3·0	3·0	1·6
12	82	0·81	0·70	29	2·54	0·48	2·8	3·1	1·5
Mean	189	0·93	0·66	76	2·43	0·46	2·67	2·65	1·44
S.E. of mean	25	0·04	0·02	13	0·11	0·02	0·13	0·08	0·03

The effects of 1 mM-dithiothreitol (10 min at pH 8·5 or 24 min at pH 7·2) in six fibres were reversed by 1 mM-5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) applied for 3 min at pH 7·2–8·5, 20 °C. The effects on amplitude and rate of decay reached a steady level in less than one min. The magnitude of the effects of DTNB in these fibres, measured at −50 mV, pH 7·2, 20 °C were: increase in amplitude of 3·1, 2·2, 2·0, 2·7, 2·2, 2·7 (mean 2·5) times; decrease in rate of decay of 2·6, 3·3, 2·9, 3·3, 2·1, 2·4 (mean 2·8) times; increase in time to peak of 1·5, 1·6, 1·2, 1·7, 1·5, 1·6 (mean 1·5) times. The magnitude of its effects were thus in the same range as those caused in the opposite direction by dithiothreitol. Records from an experiment of this kind are shown in Fig. 4.

Ben-Haim *et al.* (1973) argued that the primary action of dithiothreitol was on post-synaptic receptors rather than on the nerve terminal, because (1) miniature end-plate potentials were reduced in amplitude by low concentration (0·1 mM) of dithiothreitol, and (2) after exposure to 1 mM-dithiothreitol there was no significant change in the quantal content of end-plate potentials, measured by the failure method, in preparations paralysed by decreased Ca^{2+} and increased Mg^{2+} concentrations. In support of a primary post-synaptic action of dithiothreitol were measure-

ments of miniature end-plate currents in the present experiments. Although these were not systematically studied, it was clear that after 1 mM-dithiothreitol they were barely detectable above the noise at the standard holding potential of −50 mV. Nevertheless, after exposure to dithiothreitol miniature end-plate currents were clearly visible when the fibres were hyperpolarized, for example to −130 mV as in

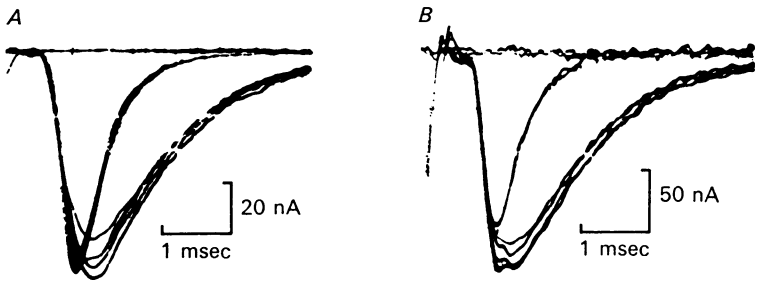

Fig. 4. Reversal of dithiothreitol actions by 1 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB). Comparison of end-plate currents in two fibres, *A* showing currents before and after dithiothreitol, *B* showing currents in a dithiothreitol-treated fibre before and after DTNB. Four superimposed currents are shown for each treatment. The slower decaying currents (before dithiothreitol in *A* and after DTNB in *B*) were recorded at 2·5 times lower amplification; fibres clamped at −50 mV.

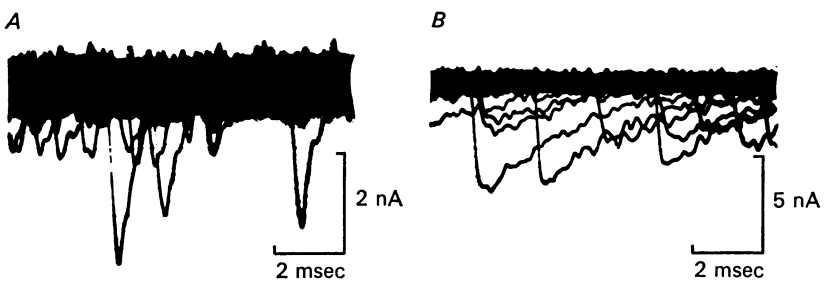

Fig. 5. Miniature end-plate currents in a dithiothreitol-treated fibre before (*A*) and after (*B*) reversal of the actions of dithiothreitol with 1 mM-DTNB. Fibre clamped at −130 mV.

Fig. 5, in which case exposure to 1 mM-DTNB increased their amplitude and slowed their decay; thus the effects of dithiothreitol on both miniature end-plate currents and end-plate currents were qualitatively similar, though a quantitative comparison was not undertaken because of the low signal to noise ratio of the miniature end-plate currents.

Effect of dithiothreitol on the relation between rate of decay of end-plate currents and membrane potential

The rate constant characterizing the exponential decay of end-plate currents in normal muscle has been shown to vary with the membrane potential (V, mV) according to the relation $\alpha(V) = Be^{AV}$ where A, mV^{-1} and B, $msec^{-1}$ are constants (Magleby & Stevens, 1972*a*, *b*; Anderson & Stevens, 1973; Dionne & Stevens, 1975). A model quantitatively consistent with the experimental observations is one where

the gating of ion channels involves a conformational change of a molecule with a dipole moment, with the component of the moment normal to the plane of the membrane changing by about 50 Debye when the channel closes (Magleby & Stevens, 1972*b*). It was of interest to determine whether this relation between rate of decay

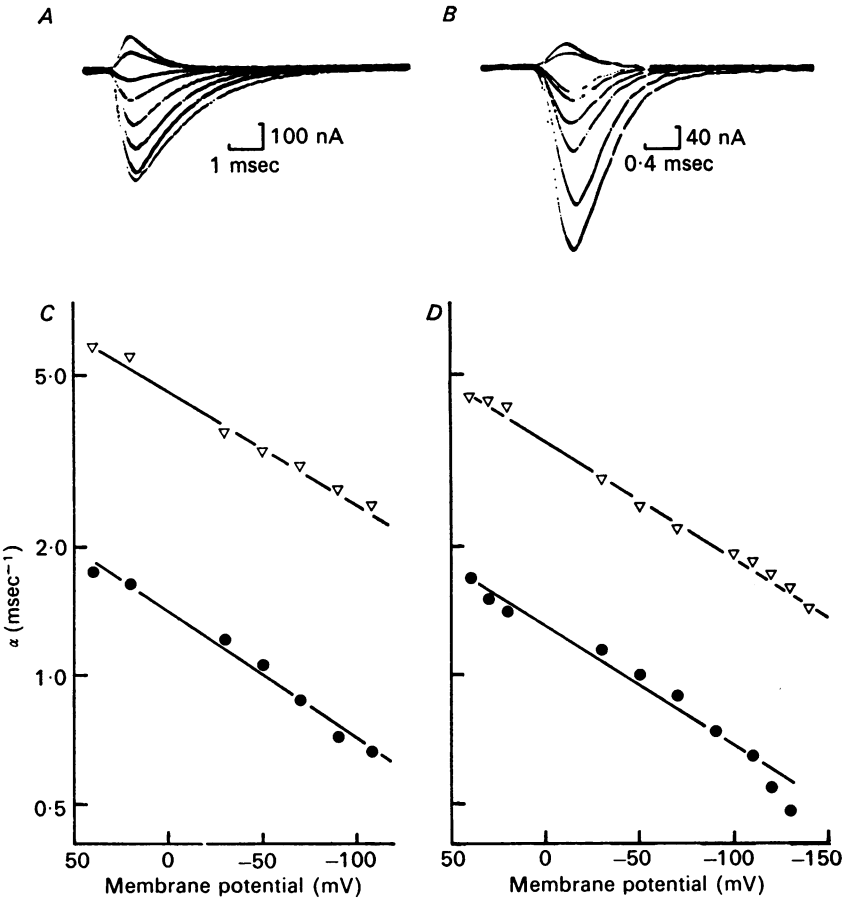

Fig. 6. Relation between ln α and membrane potential. Sample end-plate currents are shown before (*A*) and after (*B*) treatment of the fibre with 1 mM-dithiothreitol at 40, 20, −10, −30, −50, −70, −90 and −108 mV. *C* shows the relation between ln α and membrane potential plotted for the same fibre before (●) and after (▽) exposure to 1 mM-dithiothreitol, for 10 min at 20 °C, pH 8·5, but recorded at pH 7·2, each point is the mean of two to eleven currents (fifty three in total). *D* shows a similar relation in another fibre after exposure to 1 mM-dithiothreitol for 24 min at 20 °C, pH 7·2 (△), and then after exposure to 1 mM-DTNB for 3 min at pH 7·2 (●); the points are mean of five to seventeen currents. Regression lines are shown for each set of data.

of end-plate currents and membrane potential still held after the muscle had been exposed to dithiothreitol to increase the rate of decay of the currents. The procedure in experiments to test this possibility was to change the membrane potential at which the fibre was clamped by increments of 10 or 20 mV and to measure rates of decay of a series of end-plate currents at each potential; each sequence of records

always began and ended with end-plate currents at a standard potential of -50 mV. Fig. 6 shows rate constants determined from a series of end-plate currents recorded in this way, plotted semilogarithmically against membrane potential before and after treatment of the muscle with dithiothreitol. Also shown in Fig. 6*D* is the relation between ln α and membrane potential in a fibre, first treated with dithiothreitol, then with DTNB to reverse the dithiothreitol actions.

It can be seen from Fig. 6 that after dithiothreitol the rate of decay remained voltage sensitive and that the slope, A, mV^{-1}, of the relation between ln α and membrane potential was similar in controls, after dithiothreitol, or after dithiothreitol and DTNB. Values for the slopes of regression lines fitted to the data, together with 95% confidence limits were: Fig. 6*C*, before dithiothreitol 0·0068 (0·0058–0·0078 mV^{-1}), after dithiothreitol 0·0060 (0·0052–0·0068) mV^{-1}; Fig. 6*D*, after dithiothreitol 0·0063 (0·0059–0·0067) mV^{-1}, after DTNB 0·0067 (0·0058–0·0076) mV^{-1}. In ten fibres after dithiothreitol treatment the mean value of A, mV^{-1} was found to be 0·0081 (S.E. 0·0009) mV^{-1} compared with the mean value in six fibres before dithiothreitol treatment of 0·0083 (0·0009) mV^{-1}; it was concluded from two-sample t tests and Wilcoxon rank tests that the difference between these values was not significant at the 5% level.

In contrast to the small or absent effects of dithiothreitol on A, mV^{-1} were the increases observed in B, $msec^{-1}$ (the rate constant at a membrane potential of 0 mV) after dithiothreitol treatment; these would be expected in view of (1) the reported increased rates of decay of dithiothreitol-treated fibres at the standard holding potential of -50 mV (Table 1) and (2) the relation between ln α and membrane potential. In Fig. 6, values for B, $msec^{-1}$ with 95% confidence limits were: Fig. 6*C*, before treatment 1·41 (1·32–1·51) $msec^{-1}$, after dithiothreitol treatment 4·58 (4·37–4·78) $msec^{-1}$; Fig. 6*D*, after dithiothreitol 3·51 (3·39–3·63) $msec^{-1}$, after DTNB 1·31 (1·22–1·41) $msec^{-1}$. In ten fibres after dithiothreitol treatment the mean value of B, $msec^{-1}$ was 4·48 (S.E. 0·25) $msec^{-1}$ compared with a mean value in six fibres before treatment of 1·45 (S.E. 0·13) $msec^{-1}$, a change in the mean value of 3·1 times; it was concluded from both two-sample t tests and Wilcoxon rank tests that the difference between these values was significant at the 1% level.

Effect of dithiothreitol on the relation between peak end-plate current and membrane potential

In the course of the experiments just described it was noticed that when the membrane potential was made more negative than the standard potential of -50 mV, the amplitude of end-plate currents in dithiothreitol-treated fibres became larger than might have been expected if there was a linear relation between peak end-plate current and membrane potential. This effect is illustrated in Fig. 7 where peak end-plate current is plotted against membrane potential in the fibres used for the experiment on which Fig. 6 was based. It can be seen that after dithiothreitol and before DTNB (triangles) there was curvature in the relation between peak end-plate current and membrane potential, such that peak conductance seemed to increase at more negative potentials. Although a small curvature in this relation has been reported in normal muscle (without dithiothreitol treatment), it was in the opposite direction (Magleby & Stevens, 1972*b*; Dionne & Stevens, 1975) to that observed after exposure

to dithiothreitol in the present experiments; no curvature in this relation before dithiothreitol or after DTNB (circles) treatment is obvious from Fig. 7 but it remains possible that a small curvature could have been obscured by the variability of the results. The curvature in the relation after dithiothreitol treatment was consistently seen in ten fibres. The possibility was considered that this might arise from an unexpected ineffectiveness of the micro-electrode voltage clamp; however, it was found that in three fibres where end-plate currents after dithiothreitol treatment were recorded at the end-plate and after moving the electrodes 230–950 μm along the fibre, the reduced effectiveness of the clamp away from the end-plate led to less

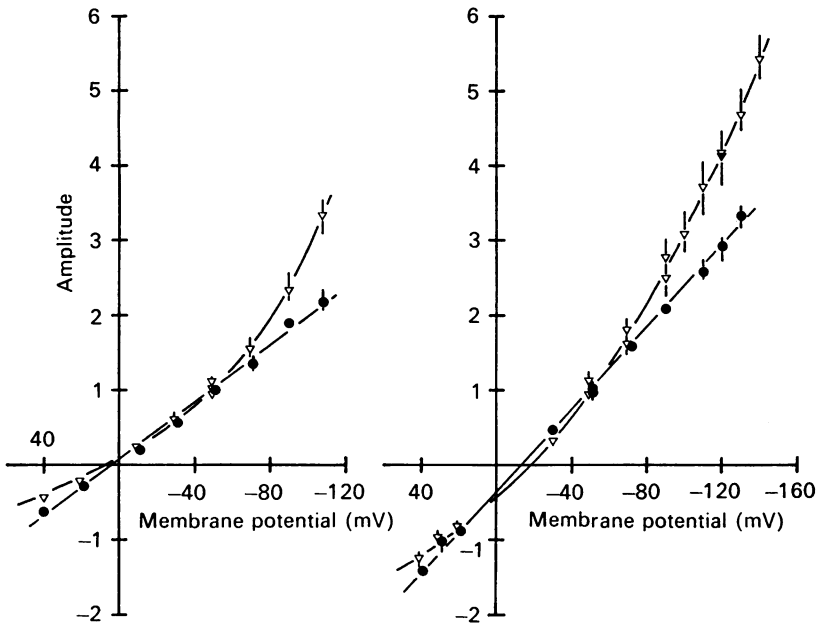

Fig. 7. Relation between amplitude of end-plate currents and membrane potential, *A* in one fibre before (●) and after (▽) treatment with dithiothreitol, *B* in another dithiothreitol-treated fibre before (▽) and after (●) reversal of the effects of dithiothreitol with DTNB. All amplitudes expressed as a fraction of the mean value at −50 mV. Points represent mean of two to seventeen currents; bars show the range. Same fibres as Fig. 6.

rather than more curvature in the relation between peak end-plate current and membrane potential. It should be mentioned that a non-linear dependence on membrane potential of the kind seen in the present experiments for end-plate currents after dithiothreitol treatment has been reported for currents evoked by ACh applied iontophoretically to end-plates of normal muscle; this was thought to arise because there was time for voltage-dependent receptor interactions to approach equilibrium during these responses (Dionne & Stevens, 1975; see also Adams, 1976; Mallart, Dreyer & Peper, 1976 and Discussion).

An incidental observation in this series of experiments was that dithiothreitol caused little or no change in the reversal potential (determined by interpolation) for

end-plate currents (see, for example, Fig. 7); this finding is in agreement with the observations of Ben-Haim *et al.* (1973) on end-plate potentials, and implies an unchanged selectivity for sodium and potassium ions of the channels opened by ACh.

DISCUSSION

Two of the main findings of the present work are the reduction by dithiothreitol in amplitude and increase in rate of decay of end-plate currents. Both these effects would be expected (see for example Gage, 1976) to contribute to the reduction in amplitude of end-plate potentials reported by Ben-Haim *et al.* (1973).

The increased rate of decay of end-plate currents after dithiothreitol is consistent with a more rapid closure of gates for ion channels associated with acetylcholine receptors. In muscle not exposed to dithiothreitol, the time constant ($1/\alpha$) for decay of end-plate currents provides a measure of the mean lifetime of ion channels opened by acetylcholine (Magleby & Stevens, 1972*b*; Anderson & Stevens, 1973), though it might be a slight overestimate in some circumstances (Katz & Miledi, 1973; Colquhoun, Large & Rang, 1977). In the present experiments dithiothreitol reduced the time constant of decay from $1{\cdot}10 \pm 0{\cdot}04$ msec (mean ± S.E., twelve muscles) to $0{\cdot}42 \pm 0{\cdot}02$ msec; this was a decrease to $38 \pm 1\%$ of the value in untreated muscle and suggested a corresponding decrease in mean channel lifetime. From noise analysis, Ben-Haim *et al.* (1975) concluded that at 20–22 °C dithiothreitol reduced mean channel lifetime from 0·94 to 0·66 msec, a decrease to $70 \pm 9\%$ of the value in untreated muscle. Differences in the potential at which the membrane was clamped do not seem to provide an adequate explanation for the greater decrease found in the present experiments, because the effect of dithiothreitol on the time constant of decay of end-plate current showed little variation with membrane potential (Fig. 6); this discrepancy thus remains for further study.

The finding that dithiothreitol treatment increased the rate of decay of end-plate currents while causing little or no change in the slope of the relation between ln (rate of decay) and membrane potential can be interpreted in molecular terms on the basis of the model proposed by Magleby & Stevens (1972*b*). Stated in terms of the Magleby & Stevens relation $\alpha(V) = Be^{AV}$ (see above), the present findings are that at 20 °C A shows little change following dithiothreitol whereas B is increased about threefold. The lack of change in A would imply that gating of ion channels associated with acetylcholine receptors continues to be controlled by the conformational change of a molecule with a dipole moment, with the change in the moment when the channel closes remaining at about 50 Debye. However, the increase in B implies that the increased rate of channel closure results from an approximately threefold increase in the free energy difference between the molecule in its 'open' (ion-conducting) conformation and in its transition state in the absence of electric field (assuming no change in the transmission coefficient of the transition, which, except in special circumstances (Laidler, 1969), is usually taken to be 1 (e.g. Laidler 1969; Mahler & Cordes, 1971; Leinhard, 1973)).

Several factors could contribute to the decrease in amplitude of end-plate currents after dithiothreitol treatment. One possibility is that dithiothreitol reduces the conductance of individual ion channels. Such an effect has been concluded from noise

analysis (Landau & Ben-Haim, 1974; Ben-Haim *et al.* 1975). Alternatively the marked increase in rate of decay of end-plate currents after dithiothreitol could lead to a reduction in their amplitude because some ion channels close before the number of open ion channels reaches a peak; this would be expected to reduce the time to peak of end-plate currents, as observed (Table 1). Such a hypothesis would also provide an explanation for the non-linear relation between amplitude of end-plate currents and membrane potential (Fig. 7) since the rate of decay, and consequently its effect on amplitude, decreases at more negative membrane potentials. This explanation is similar to that proposed by Dionne & Stevens (1975; see also Adams, 1976, and Mallart *et al.* 1976) to account for the non-linear relation between current responses to iontophoretically applied ACh and membrane potential. The curvature after dithiothreitol is analogous to that observed with iontophoretic application of ACh to normal muscle in the sense that in both cases the duration of application of ACh is relatively long compared with channel lifetimes, in the first case because of increased rates of channel kinetics, and in the second because of the prolonged duration of ACh application. This argument implies that in the conditions of the present experiments the opening and closing of ion channels begins to approach equilibrium even during the brief pulse of ACh released from the nerve.

Although this hypothesis is in line with the proposed mechanism of action of dithiothreitol, another possibility which cannot be excluded on the basis of the present evidence is that individual ion channels display rectification after dithiothreitol. It should be mentioned that a consequence of differences in the current–voltage relation before and after dithiothreitol treatment is that the extent of the dithiothreitol effect on amplitude of end-plate currents decreases as the membrane potential becomes more negative.

A factor which could contribute to the reduction in amplitude of end-plate currents is a change in the binding properties of receptors. An analogy can be drawn between the present results and those of Colquhoun *et al.* (1977) who found that false miniature end-plate currents caused by quanta of acetylmonoethylcholine were smaller than normal ones, apparently because the affinity of acetylmonoethylcholine is lower than that of ACh. If dithiothreitol were to decrease the affinity of ACh for receptors, the amplitude of end-plate currents should be decreased, as observed. It should also be noted that a further parallel between the present results and those of Colquhoun *et al.* (1977) is that the rate of decay of these false miniatures (like that for end-plate currents after dithiothreitol) is faster than that of miniature end-plate currents in untreated muscle.

From the preceding discussion it appears that at the molecular level the actions of dithiothreitol arise from the modification of a gating molecule for ion channels opened by ACh and/or of the receptor (defined as binding a molecule of ACR) though in pursuing this argument it must be remembered that a single macromolecule might perform both functions. Four lines of evidence suggest that at least part of the action of dithiothreitol is on receptors: (1) dithiothreitol changes the effectiveness of some antagonists such as (+)-tubocurarine which bind to receptors (Rang & Ritter, 1971; D. A. Terrar, unpublished observations); (2) it was concluded from analysis of dose response curves of frog muscle exposed to iontophoretically applied ACh that receptor reduction by dithiothreitol increased the equilibrium

dissociation constant for ACh (Ben-Haim *et al.* 1975); (3) the effect of dithiothreitol on end-plate currents in the presence of the anti-cholinesterase neostigmine was consistent with a reduction in the affinity of receptors for ACh released from the nerve (Terrar, 1976); (4) after dithiothreitol, some cholinergic ligands alkylate nicotinic receptors in *Electrophorus* electroplaques and have been used in receptor isolation (Karlin *et al.* 1973). As discussed above, the rate of decay of end-plate currents reflects gating of ion channels, whereas the amplitude is at least partly determined by the ACh-binding properties of receptors. In the present experiments, the actions of dithiothreitol on rate of decay and amplitude developed concurrently (see Figs. 2 and 3) as first order processes; the simplest interpretation of these findings is that reduction of a single class of disulphide bonds results in the two effects. Indeed, all the actions of dithiothreitol are consistent with a model in which dissociation of ACh from receptors accompanies closure of ion channel gates. In terms of this model, reduction of disulphide bonds by dithiothreitol increases the rate of dissociation of ACh from receptors leading to (1) an increased rate of closure of gates for ion channels which accounts for the increased rate of decay of end-plate currents, and (2) a decreased affinity of receptors for ACh which (at least partially) accounts for the reduced amplitude of end-plate currents. If the binding of ACh to receptors in their 'open' state is a two-stage process (Magleby & Stevens, 1972*b*) the overall dissociation rate constant of ACh from this open conformation would include the rate constant for isomerization to the closed state.

One puzzling observation not yet considered is the finding that decay of end-plate currents followed a simple exponential time course throughout the onset of action of dithiothreitol. One possibility is that the gating of each ion channel is influenced by the existence of several similar disulphide bonds which are reduced in turn, each stage of reduction being associated with a progressively shorter mean channel lifetime; in such a case end-plate current decay at an intermediate level of dithiothreitol action might represent the sum of several exponentials with rate constants differing by less than threefold, the sum being indistinguishable with the present methods from a simple exponential. Such a situation might arise if each ion channel were controlled by several receptors, each containing a reducible disulphide bond; this explanation provides a further speculative parallel with biochemical experiments where receptor sub-units have been described (Biesecker, 1973; Hucho & Changeux, 1973), and with experiments showing a co-operative action of ACh on receptors (Katz & Thesleff, 1957; Rang, 1971; Jenkinson & Terrar, 1973; Adams, 1975; Dreyer & Peper, 1975; Hartzell, Kuffler & Yoshikami, 1975; Magleby & Terrar, 1975).

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