The Paralytic Agent 2,4-Dithiobiuret Decreases Open Time of Murine Skeletal Muscle Acetylcholine Receptor Channels

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ABSTRACT

2,4-Dithiobiuret (DTB) causes a delayed-onset neuromuscular weakness when given chronically to rats. Mechanisms underlying this effect involve disruptions of acetylcholine (ACh) release and possibly effects on the ACh receptor channel complex. Previous experiments demonstrated a decrease in decay time constants for end-plate currents and miniature end-plate currents of muscles from rats exhibiting DTB-induced muscle weakness compared with those of controls. The purpose of the present study was to determine whether the alteration in rise and decay times for synaptic currents was due to direct effects of DTB on ACh receptor channels. Currents carried through single ACh-activated channels were recorded using patch voltage-clamp techniques in G8 mouse myotubes exposed to DTB in their growth medium and from intact hemidiaphragm preparations of rats treated with DTB by examining fluctuations in membrane noise during iontophoresis of agonist. Exposure of myotubes to DTB (1 or 10 μM) decreased the mean channel open time induced by suberyldicholine for short durations of exposure, whereas longer exposures (24–48 h) to DTB were required in order for decreased open times for ACh as an agonist to be observed. In the absence of DTB, closed times for single channels of G8 cells were described by a two-exponential fit reflecting intraburst and interburst closures. At 1 μM DTB, the duration of gaps within bursts and of gaps between bursts increased. Similarly, for hemidiaphragm preparations taken both from rats that were treated for 7 days with DTB at 1 mg/kg/day (a dosing regimen that causes muscle weakness) and from rats that were treated acutely with 1 mg/kg and the diaphragm removed 24 h later, mean open times derived from fluctuations of end-plate current noise were reduced. These decreases in channel open time may underlie the decrease in the decay time constant for MEPCs observed in muscles taken from rats treated with DTB.

Daily treatments of rats with the thiourea derivative DTB at doses ranging from 0.25 to 2 mg/kg/day leads to development of an ascending neuromuscular weakness after a delay of 4 to 16 days, depending on the daily dose (Atchison and Peterson, 1981; Atchison et al., 1981). Electrophysiological studies characterizing the DTB-induced muscle weakness have demonstrated decreased quantal release of ACh from motor axon terminals, observed as decreased quantal content of EPPs and decreased frequency of both spontaneously occurring and depolarization-evoked MEPPs at end-plates of hindlimb muscles at the time of neuromuscular weakness (Weiler et al., 1986; Atchison, 1989). In addition, there is an increased incidence of large-amplitude MEPPs with slow rise and decay times, and the mean rise and decay times for all MEPPs appear to be shifted to longer times in muscles from DTB-treated rats (Atchison, 1989).

The results of two microelectrode voltage-clamp experiments revealed that at least three different populations of quanta occurred at neuromuscular junctions from rats treated with DTB (Spitsbergen and Atchison, 1991). There was an increase in the occurrence of large-amplitude MEPCs with slow rise and decay times and an increase in the occurrence of very small MEPCs. In all treated preparations, MEPCs were observed that had fast rise and decay kinetics. The τ for the fast-rise-and-decay MEPCs from treated preparations was decreased compared to similar MEPCs from control preparations (Spitsbergen and Atchison, 1991). The neuromuscular weakness observed in rats treated

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ABBREVIATIONS: DTB, 2,4-dithiobiuret; DTT, dithiothreitol; ACh, acetylcholine; AChR, acetylcholine receptor; MEPP, miniature end-plate potential; EPP, end-plate potential; MEPC, miniature end-plate current; EPC, end-plate current; τ, decay time constant; SCh₂, suberyldicholine; CCh, carbamylcholine.
chronically with DTB is accompanied by electrophysiological changes in transmission that are similar in many ways to those observed in human patients suffering from a variety of neuromuscular disorders (Engle et al., 1977; 1982; 1990). Similarities include decreased quantal content for nerve-evoked EPPs and EPCs, decreased MEPP amplitude, slowing of the MEPP decay and a more rapid decay of synaptic transmission. When at rest, but during even moderate activity they exhibit increasing degrees of skeletal muscle weakness. A similar reduction in safety factor and abnormal quantal secretion have been detected before the onset of muscle weakness in neuromuscular preparations from rats treated with DTB (Atchison, 1990). By understanding how DTB interacts with different components of junctional transmission and how such interactions lead to the weakness observed in treated rats, we may gain a better understanding of the causes of weakness in some of these human disorders.

The purpose of this study was to determine whether the decrease in $\tau_{\text{MEPC}}$ and $\tau_{\text{MEPP}}$ observed at end-plates of rats treated with DTB is due to a direct effect of DTB on current flow through nicotinic AchR-activated channels. To answer these questions, we studied membrane noise induced by iontophoretic application of Ach in hemidiaphragm preparations taken from control rats and exposed to DTB in the bathing medium and from rats 24 h and 7 days after treatment with DTB. Single-channel currents were recorded using patch voltage-clamp techniques with a clonal cell line (G8) exposed to DTB in the growth medium. Analysis of single-channel open and closed times via patch voltage-clamp and fluctuation analysis indicated that exposure to DTB decreased single-channel open time.

Materials and Methods

Chemicals. The following agents were used in this study: purified, recrystallized DTB (Ash Stevens, Detroit, MI), acetylcholine, suberyldicholine and EGTA (Sigma Chemical Co., St. Louis, MO), HEPES (U.S. Biochemicals, Cleveland, OH).

Cell culture methods. Mouse G8 myoblasts (passage 20–25) were used for all patch voltage clamp studies. These cells express muscle-type nicotinic AchR, which have been well characterized electrophysiologically (Morris and Montpetit, 1986; Morris et al., 1989). Cells were obtained from the American Type Culture Collection (Rockville, Maryland) and were grown in collagen-coated 30-mm culture dishes and maintained in Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum (Gibco) and 10% horse serum (Gibco). Differentiation of myoblasts was enhanced by growth in normal growth medium until confluence and then decreasing the serum content to 2% fetal bovine serum and 2% horse serum. After a decrease in the serum content, these cells differentiate to form multinucleated myotubes, which are highly responsive to Ach. Electrophysiological recordings were made from myotubes within 3 to 4 days of differentiation.

Exposure of cells in culture to DTB. Two protocols were used for exposure of cells to DTB in these experiments. In the first, patches were excised from control cells and single-channel currents were recorded in the presence of agonist (0.1–2 $\mu$M Ach or 0.1 $\mu$M SCh$_{2}$), followed by exposure to agonist plus DTB (1–1000 $\mu$M). In the second protocol, cells were grown in medium containing DTB (1–1000 $\mu$M). After exposure of cells to DTB for 1 to 3 days, single-channel currents were recorded in the presence of 0.1 to 2 $\mu$M Ach. The rationale for the choice of concentration range of DTB used was that it is very close to the concentration of DTB-derived equivalents of $^{14}$C measured in plasma of rats treated with 1 mg/kg/day of $^{14}$C-DTB (Porter et al., 1983). Moreover, we demonstrated previously that abnormal MEPCs are observed as early as 24 h after treatment with 1 mg/kg of DTB and within 4 h of exposure of control hemidiaphragms to 1 $\mu$M DTB (Spitsbergen and Atchison, 1990).

Intact neuromuscular preparations. All fluctuation analysis studies were performed on hemidiaphragm preparations taken from control rats and from rats treated previously with DTB. Male Sprague-Dawley rats (200–220 g, Harlan Industries, Madison, WI) were used. One group of rats was treated for 7 days with 1 mg/kg/day i.p. of DTB, a treatment regimen that reliably causes muscle weakness (Atchison and Peterson, 1981). A second group of rats was given a single 1-mg/kg dose of DTB, and hemidiaphragms were removed 24 h later, at which time no muscle weakness was observed, to study early effects of DTB that preceded muscle weakness (Spitsbergen and Atchison, 1990). Several muscles taken from control rats were exposed to 1 $\mu$M DTB for up to 4 h in the bathing medium to determine whether acute exposure to DTB alters AchR channel function. Otherwise, all recordings were performed in the absence of DTB to ensure that the effects observed were due to the treatments described. As noted above, our previous studies indicated that abnormal MEPCs occurred as early as 24 h after treatment with 1 mg/kg of DTB and within 4 h of exposure of control hemidiaphragms to 1 $\mu$M DTB (Spitsbergen and Atchison, 1990).

Electrophysiological methods. Single AchR channel currents were recorded from G8 myotubes using low-resistance, fire-polished microelectrodes fabricated from borosilicate glass (1-mm outside diameter, WPI Instruments, New Haven, CT). Currents were recorded using standard patch voltage-clamp techniques on cell-free patches in the "outside-out" configuration (Hamill et al., 1981). During electrophysiological recording, the bathing medium was changed to a physiological saline; it contained (in mM) NaCl, 135; KCl, 5; MgCl$_2$, 1; CaCl$_2$, 2; d-glucose, 11; HEPES, 14 (set to pH 7.3 with NaOH). Patch electrode (5–10 M$\Omega$ resistance) solutions contained (in mM): KCl, 135; MgCl$_2$, 2; d-glucose, 10; HEPES, 10; ethylene glycol-bis (β-amino ethyl ether) N,N'-tetraacetic acid (EGTA), 2 (set to pH 7.3 with KOH). All solutions used were filtered (0.22-μm pore size) before use. Single-channel currents were filtered at 2 kHz, amplified 100× using an Axopatch-1D patch clamp amplifier (Axon Instruments, Foster City, CA) and recorded to FM tape (15 inches per second, Store 4DS, Racal Recorders Inc., Irvine, CA). During analysis, current recordings were sampled at 50-μs intervals, stored on hard disk and analyzed off-line using the pClamp® analysis software (Axon Instruments, Foster City, CA) on a Zentith Microcomputer (model ZW-249–84). The opening and closing of channels was detected by a half-amplitude current threshold (Colquhoun and Sigworth, 1985). Because of the limited bandwidth of the data analysis system, the minimum duration of event that was detectable was 100 μs. Histograms of open and closed times were constructed from the idealized open and closed intervals. Mean values for open and closed durations were determined from exponential curves fit to the distributions. For the determination of closed duration, only recordings containing a single active channel were used. Recordings containing more than one active channel were analyzed for open duration, but overlapping channel events were not included in this analysis. The mean amplitude of channel openings was determined from gaussian distributions fit to histograms of channel amplitudes recorded for a given patch. Channel conductance was determined from the slope of the current-voltage relationship for a given amplitude class of channels. In most patches, multiple conductance levels were observed, but channel openings with a slope conductance of 37 pS.
predominated. Only this conductance class was used in further analysis.

Fluctuation analysis studies were performed using two microelectrode voltage-clamp techniques (Takeuchi and Takeuchi, 1959) and hemidiaphragm preparations from control rats and from rats treated previously with DTB. Voltage-recording and current-passing electrodes had impedances of 5 to 10 MQ and 2 to 4 MQ, respectively, when filled with 3 M KCl. Lower-resistance microelectrodes were used for control passage; this allowed passage of the large amounts of current necessary to maintain space-clamp of the end-plate region of the muscle cells. By passing low levels of positive current (2–10 pA) through the iontophoretic electrode (resistance of 50–100 MQ when filled with 2 M ACh), it was possible to elicit EPCs of 2–20 nA, which could be maintained for many seconds (up to 60 s). To prevent ACh from leaking from the electrode when not iontophoresing, a negative retaining current was passed (2–4 pA). Membrane noise in the absence and presence of ACh was recorded at 1 to 4 different end-plate regions in each hemidiaphragm preparation at holding potentials ranging from −20 to −70 mV. All signals containing noise before and after iontophoresis of ACh were recorded to FM tape (15 ips, Store 4DS, Racal Recorders Inc., Irvine, CA) for storage before analysis. During analysis, noise-containing signals were sampled at 50-s intervals, stored on hard disk and analyzed off-line using the program SPAN (Spectral Analysis Program, J. Dempster, University of Strathclyde, Glasgow) on a Compaq microcomputer. For analysis, two channels of noise-containing signal were recorded to computer. One was DC-coupled, amplified 1 to 10 times and used to determine the amplitude of the iontophoretic current and to edit out signals containing spontaneous synaptic currents; the other was AC-coupled and amplified 100 to 1000 times. This channel was used to determine the amplitude and duration of the noise fluctuations in the absence and presence of iontophoresed ACh. The AC-coupled signal was low-pass (500 Hz) and high-pass (1 Hz) filtered (modified Butterworth response) using an electronic filter (VBP/8 dual-channel variable filter, Kemo Limited, Kent, UK). Power spectra were generated from each end-plate region before and after iontophoresis of ACh. Background spectra were subtracted from spectra containing information describing ACh-elicited currents. The decay time constants for channels were determined from the corner frequency ($f_c$) of the calculated spectra (Anderson and Stevens, 1973).

**Statistical analysis.** Effects of DTB on channel burst duration as a function of time and holding potential were examined using a repeated-measures analysis of variance (Steele and Torrie, 1960). For comparison of single-time-point effects of DTB, Student's $t$ test was used (Steele and Torrie, 1960). Post hoc comparisons were made using Bonferroni's test. For all comparisons, differences were considered statistically significant at $P < .05$.

**Results**

**Results of Patch-Clamp Studies**

Effects of DTB on open and closed durations. The first experiments performed examined the effects of acute exposure to 1 μM DTB on the open time of single AChR-activated channel currents. Open-time histograms were fit with exponential functions that were the sum of two exponentials. The top panel shows single-channel currents elicited by 100 nM Sc2+ recorded from an "outside-out" patch held at −100 mV and an open-time distribution from a patch excised from a control cell. The bottom panel shows the effect of DTB on fast and slow $\tau$. Data shown represent the mean ± S.E.M. of currents recorded from patches of control cells from 12 dishes and currents from cells exposed to DTB in six dishes. The asterisk (*) denotes values significantly different from control at $P < .05$.

**Fig. 1.** Effects of acute exposure to 1 μM DTB on the open time of single AChR-activated channel currents.
exposure of G-8 cells to DTB using ACh as agonist. The results of these experiments suggested that DTB may decrease channel open time, but the changes observed were quite small (results not shown). Thus we changed the agonist used to SCh₂, which binds to the AChR with a greater affinity than ACh and therefore maintains the channel in an open state for longer periods of time (Marshall et al., 1991). We hoped that prolonging channel open time by using SCh₂ as an agonist would make changes in open time after exposure to DTB more evident. When SCh₂ was used as an agonist, the distribution of channel open times was described by two exponentials. Figure 1 displays the effect of exposure to 1 μM DTB on channel open time. As early as 5 min after exposure to 1 μM DTB, alterations in open time can be detected. This is observed as a decrease in the slow phase of open time. After 30 min of exposure to DTB, fast \( \tau \) was decreased significantly for channels opening in the presence of 100 nM SCh₂, whereas slow \( \tau \) was statistically unchanged (fig. 1). Exposure of cultures to DTB for extended periods of time altered channel open times to a point at which changes could be detected when ACh was used as agonist. In contrast to channel openings elicited by SCh₂, the distributions of channel openings elicited by ACh were described by a single exponential. Figure 2 shows that growth of myotubes for 24 to 48 h in the presence of 1μM DTB leads to a decrease in \( \tau \) calculated for channel openings elicited by 100 nM ACh.

Alterations in the duration of channel closures may yield even more information than changes in channel open times about the mechanism of action of compounds that alter channel function. When we analyzed channel closed times in the absence of DTB, we found that the distribution of closures was described by the sum of two exponentials. The rapid component of closures represents brief closures within bursts, whereas the slow component represents closures between channel openings. Maintenance of myotubes in growth medium containing 1 µM DTB caused an increase in the duration of gaps between bursts of channel activity (fig. 3).

Results of Fluctuation Analysis Studies

Fluctuations in iontophoretic current. After a change in the current flowing through the iontophoretic electrode from \(-4\) pA to \(+10\) pA, a current with amplitude of 5 to 50 nA can be measured at the end-plate (results not shown.). The number of channels that open at equilibrium, during ionto-
phoresis is not constant but fluctuates about an average (see Anderson and Stevens, 1973). If the signal is amplified, this represents the opening and closing of channels at the end-plate region. After transformation of the signal using a fast Fourier transform, a power spectrum can be generated and then fit with a Lorentzian function, similar to the fit of single-channel events with exponential functions. The decay time constant $\tau$ for channels generating the noise can be determined from the corner frequency ($f_c$) of the spectra (or Lorentzian function); $f_c$ is the frequency at which the power in the components of the signal is one-half of the maximum power. From $f_c$, the time constant $\tau$ can be calculated using the equation $\tau = 1/2\pi f_c$ (Anderson and Stevens, 1973). Figure 4 depicts the effects of DTB treatment on channel open time for end-plate regions of muscles taken from control rats and from rats treated for 7 days with 1 mg/kg of DTB. Open time for channels in muscles taken from rats that exhibit muscle weakness after chronic treatment with DTB is decreased at holding potentials of $-20$, $-50$ and $-70$ mV. However, DTB did not significantly reduce open times at $-20$ mV for any treatment paradigm, whereas at $-70$ mV both acute and chronic exposure to DTB reduced burst duration. At a holding potential of $-50$ mV, the longer durations of exposure led to significant reductions in the duration of channel open times (fig. 4).

**Discussion**

**Effects of DTB on single AChR-channels.** Results of previous voltage-clamp experiments in which $\tau_{PC}$ and $\tau_{MEPC}$ were found to be decreased in muscles from rats treated with DTB (Spitsbergen and Atchison, 1991) suggested possible postsynaptic effects of DTB. However, currents recorded in these studies were due to the release of ACh from the presynaptic terminal. Thus alterations in release processes or junctional morphology could alter these currents. In the studies described herein, we examined channel behavior in the presence of exogenously applied ACh, which enabled us to examine changes in postsynaptic function in isolation. When we examined the effects of DTB on channel behavior, we found that $\tau$ values calculated from muscles taken from rats exhibiting muscle weakness after treatment with 1 mg/kg/day of DTB for 7 days were decreased compared to $\tau$ values from control muscles. Similar effects on $\tau$ were observed in muscles taken from rats treated 24 h before with a single dose of DTB (1 mg/kg) (Spitsbergen and Atchison, 1991). Application of 1 $\mu$M DTB in the bathing medium to muscles from control rats also appears to affect $\tau$ (at hyperpolarized holding potentials). Thus the bursts of AChR channel activity observed after iontophoresis of ACh onto muscles of rats treated with DTB are shorter in duration than those observed in muscles of control rats. When effects are monitored at the level of the single channel using patch voltage-clamp techniques on cultured myotubes, changes in open time are observed as early as 30 min after exposure to DTB (1 $\mu$M) and possibly as early as 5 min after exposure. The early times at which these effects are observed indicate that changes in neuromuscular function begin to occur soon after exposure to DTB is initiated. Perhaps these alterations are in part responsible for the weakness observed with continued exposure.

**Block of open channels by DTB.** Many compounds block the nicotinic AChR channel after its opening. All of the agonist molecules tested thus far have been found to block the channel (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985; Marshall et al., 1991). Classical competitive receptor blockers such as d-tubocurarine also block the ion channel (Colquhoun et al., 1979), as do a variety of other charged and uncharged molecules (Neher and Steinbach, 1978; Ogden et al., 1981; Neher, 1989). In the presence of open-channel
were generated by fast Fourier transform of the recorded signal. The corner frequency (of ion channels open becoming blocked and unblocked in rapid succession. The bursts of activity, which represent channels opening and blockers, the single-channel currents exhibit characteristic bursts of activity, which represent channels opening and becoming blocked and unblocked in rapid succession. The effects of open-channel block on EPCs and MEPCs are observed as a biphasic decay of the current. The normal number of ion channels open after the release of transmitter into the synaptic cleft, so the initial amplitude of the current is normal. Soon after opening, however, a portion of the channels become blocked. This leads to a very rapid initial decay of the current. The prolonged phase results from channels becoming unblocked over time and allowing current to flow through them before their final closure (Adams and Sakmann, 1978; Lambert et al., 1980).

Effects on the affinity of receptors for agonist. Previous studies have demonstrated the presence, within the receptor region of the AChR channel complex, of disulfide bonds that are critical to normal function. The reduction of disulfides present on the receptor via dithiothreitol (DTT) decreases the responsiveness to ACh, whereas reoxidation via 5,5'-dithio-bis (2-nitrobenzoate) reverses these effects when examined in Torpedo electroplax preparations (Karlin and Winnik, 1968) and chick muscle (Rang and Ritter, 1971). Walker et al. (1981) examined both agonist binding and ion flux in membrane-bound vesicles containing AChRs from T. electroplax. They observed that reduction of the receptors with DTT decreased the binding affinity of AChRs for CCh and shifted the dose-response curve for CCh-induced increases in 22Na+ permeability to higher CCh concentrations. Effects of thiol-group modification on ion flux activation and inactivation kinetics were examined further by Walker et al. (1984). In these studies, CCh binding and 86Rb influx into vesicles with reconstituted AChR channels purified from Torpedo californica were measured before and after reduction with DTT. The main effect of DTT reduction was to shift EC50 values for activation and slow inactivation to higher agonist concentrations. These findings are consistent with a decrease in binding affinity for CCh previously described by this group. Electrophysiological studies performed on muscles exposed to DTT have demonstrated that after exposure to DTT,
the amplitude and decay times for EPPs and MEPPs were decreased (Ben-Haim et al., 1973; Terrar, 1978). Studies utilizing fluctuation analysis demonstrated that after reduction of AChRs with DTT, the time that single channels remain open and the conductance for these channels are decreased (Ben-Haim et al., 1975). Thus reduction of critical disulfide groups located on the AChR leads to a decrease in affinity of the receptor for agonist, and associated with reduction is a decrease in single-channel conductance and open time. It is possible that the effects of DTT, which is a moderate disulfide-reducing agent (Preisler and Bateman, 1947), on channel open time are due to reduction of disulfide groups in a manner similar to that observed after reduction with DTT. Previous studies have indicated that alterations in thiol/disulfide status within cells may be related to toxic effects of DTT (Williams et al., 1986).

In conclusion, the results described above indicate that exposure of AChR channels to DTT alters these channels in such a way as to decrease the time they remain open. There are two alternative conclusions that could be drawn from these results. One is that the decrease in single-channel open time has no pathophysiological significance in the genesis of the muscle weakness, because it is observed in rats that exhibit weakness as well as in those that display no signs of weakness. Alternatively, we suggest that these effects are some of the earliest changes observed after exposure to DTT and that continuous alteration in these processes bring about changes that lead to the observed weakness. Thus DTT has very early and direct effects on AChR-activated channels. These effects occur at dosage levels the same as or less than those at which changes in ACh release occur.

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