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MODIFICATION OF IONIC CONDUCTANCE AT THE NEUROMUSCULAR JUNCTION FOLLOWING EXPOSURE TO THE PARALYTIC AGENT 2,4-DITHIOBIURET.

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MODIFICATION OF IONIC CONDUCTANCE AT THE NEUROMUSCULAR JUNCTION FOLLOWING EXPOSURE TO THE PARALYTIC AGENT 2,4-DITHIOBIURET

By

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ABSTRACT

MODIFICATION OF IONIC CONDUCTANCE AT THE NEUROMUSCULAR JUNCTION FOLLOWING EXPOSURE TO THE PARALYTIC AGENT 2,4-DITHIOBIURET

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Rats treated with small daily doses of 2,4-dithiobiuret (DTB) develop a delayed onset neuromuscular weakness following 4 to 6 days of treatment. Analysis of quantal release of acetylcholine using nerve-muscle preparations taken from rats exhibiting muscle weakness following chronic treatment with DTB, demonstrated a decrease in quantal content of evoked end-plate potentials, a decrease in the frequency of spontaneously occurring miniature end-plate potentials and a prolongation of rise and decay times for end-plate potentials and miniature end-plate potentials. The studies described herein were designed to answer two questions. First, at what time following exposure to DTB can the earliest alterations in transmission be detected? Second, can the alteration of rise and decay times for synaptic potentials be attributed to effects of DTB on current flowing through acetylcholine receptor-gated channels at the neuromuscular junction? To answer these questions synaptic potentials, synaptic currents and membrane noise induced by iontophoretic currents were studied in hemidiaphragm preparations from rats 1, 4, 8, 24 hours and 7 days following treatment with DTB, or from control rats and exposed to DTB in the bathing medium. Single-channel currents were recorded using patch voltage clamp techniques with a clonal cell (G8) line exposed to DTB

in the growth medium. Results of these studies indicate that transmitter release is altered in hemidiaphragm preparations taken from rats as early as 1 hour after treatment, or within minutes following exposure of control muscles to DTB in the bathing medium. Kinetic analysis of the decay of synaptic currents in muscles from rats treated with DTB and in muscles from control rats exposed to DTB in the bathing medium revealed a shortening in the decay of these currents. Analysis of single-channel open- and closed-times using patch voltage clamp and fluctuation analysis, indicated that exposure to DTB decreased single-channel open-time. In conclusion, soon after exposure to DTB is initiated alterations in both transmitter release and the kinetics of current decay can be detected. These alterations, many of which are observed in muscles taken from chronically treated rats exhibiting muscle weakness, appear to be early effects of DTB, which with continued exposure could lead to the observed weakness.

Dedicated to my wife Sandy and my parents for all their help and support.

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LIST OF ABBREVIATIONS

Acetylcholine	ACh
Acetylcholine receptor	AChR
Carbamylcholine	CCh
Corner frequency	F_c
Decay time constant	τ
2,4-Dithiobiuret	DTB
5,5'-dithio-bis (2-nitrobenzoate)	DTNB
Dithiothreitol	DTT
End-plate potential	EPP
End-plate current	EPC
Ethylene glycol-bis-(ß-amino ethyl ether) N,N-tetraacetic acid	EGTA
Gigaohm	GΩ
Hertz	Hz
Intraperitoneal	ip
Megohm	MΩ
Micromolar	μM
Millivolt	mV
Miniature end-plate current	MEPC
Miniature end-plate potential	MEPP
Nanoampere	nA
N-hydroxyethylpiperazine-N'-2-ethane sulfonic acid	HEPES
Picoampere	pA
Quantal content	m
Rate constant for channel closing	α
Suberyldicholine	SCh_2

INTRODUCTION

The studies described in the following dissertation were designed to examine alterations in transmission at the neuromuscular junction of rats treated with the paralytic agent 2,4-dithiobiuret (DTB). Rats treated with small daily doses of DTB (1 mg/kg/day) become paralyzed following approximately 1 week of treatment, while rats treated acutely with a single large dose of DTB do not exhibit muscle weakness. Results of previous intracellular recording studies indicate that transmitter release is depressed in neuromuscular preparations taken from rats exhibiting muscle weakness following chronic treatment with DTB (Weiler et al., 1986; Atchison 1989). This depression of release is observed as a decrease in quantal content of evoked end-plate potentials and a decrease in the frequency of spontaneous and depolarization evoked miniature end-plate potentials (Weiler et al., 1986; Atchison 1989). Alterations in transmitter release such as these are normally indicative of a presynaptic site of action. What was not known, at the time when the studies described herein were initiated, was whether alterations in transmission occur early on during treatment of rats with DTB and progress to the observed weakness, or whether effects on transmission are only observed once weakness is observed. It was

also not known whether an alteration of postsynaptic processes was related to the weakness observed in treated rats. The purpose of the studies described herein was to determine whether early effects on transmitter release could be detected in rats treated with DTB and to explore the possibility that a postsynaptic effect of DTB was related to the observed weakness.

Chapter 1. The neuromuscular junction.

The synapse formed between motor nerves and muscle cells is known as the neuromuscular junction. Axons innervating muscles normally branch prior to synapsing on a muscle fiber. Thus each axon innervates more than one fiber. At mammalian neuromuscular junctions each fiber is innervated at only one point by a single nerve. The synapse formed at neuromuscular junctions is composed of a presynaptic terminal containing transmitter-filled vesicles and a postsynaptic component with a large number of receptor molecules. In vertebrates these vesicles contain the neurotransmitter acetylcholine (ACh). ACh is synthesized in the nerve by choline acetyltransferase from the precursors choline and acetyl coenzyme A. Following synthesis, ACh is stored within synaptic vesicles prior to its release. These vesicles are found throughout the terminal but normally aggregate around presynaptic densities, termed active zones. These densities appear as fuzzy dense projections in most ultrathin sections viewed using a transmission electron microscope. A much clearer picture of the organization of these particles is obtained by using the freeze-fracture technique. Utilizing this method the presynaptic particles appear as parallel lines of intramembranous proteins located across the synaptic cleft from postsynaptic active regions. Recent evidence indicates that these protein molecules may be calcium-channels important for calcium (Ca^{++}) influx during transmitter release (Heuser et al., 1974). Terminals also contain large numbers of mitochondria and cisternae of smooth endoplasmic reticulum (Figure 1).

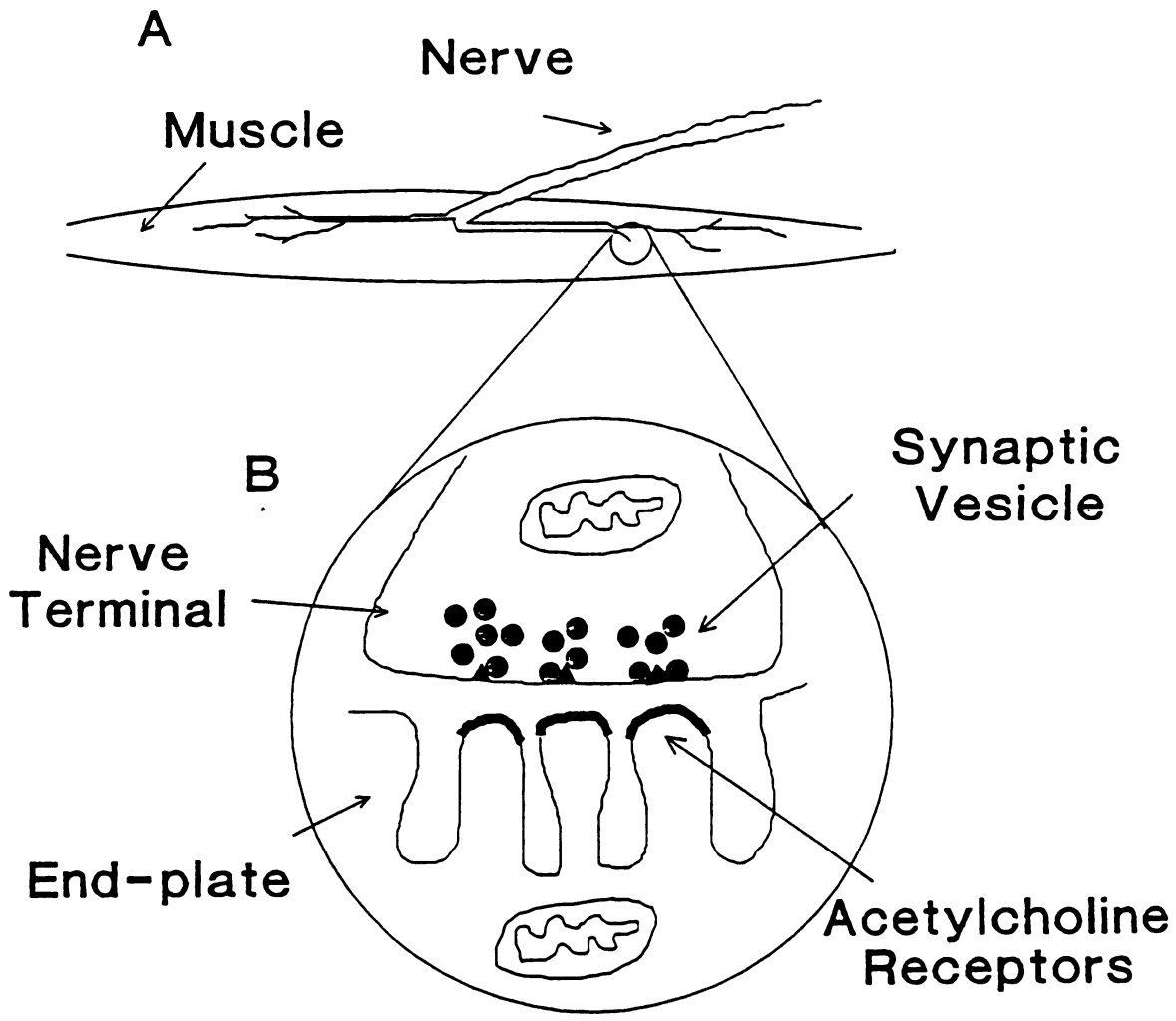


Figure 1. Innervation of Muscles.

Each muscle cell is innervated by one or more nerves, each nerve normally branches to innervate more than one muscle fiber and each muscle fiber is normally innervated by a single nerve.(a). The point at which a muscle fiber is contacted by a nerve is known as an end-plate region (b). Located within the nerve terminal are a number of synaptic vesicles containing neurotransmitter (acetylcholine). The end-plate region is highly folded and contains a large number of receptors which are located at the tops of the membrane folds.

These cisternae are often associated with coated vesicles and a small number of dense-cored vesicles. Experiments monitoring the uptake of horseradish peroxidase into terminals following nerve stimulation indicate that the smooth endoplasmic reticulum cisternae and coated vesicles are involved in the reformation of synaptic vesicles following transmitter release (Heuser and Reese, 1973).

The region of the muscle at which the nerve makes contact is termed the sole-plate, or end-plate region. At this site, there are several muscle cell nuclei and large numbers of mitochondria, all located beneath a highly folded region of the muscle cell membrane. If one examines sections through a terminal and its associated postsynaptic structures it can be seen that the folds in the muscle cell membrane occur beneath the presynaptic active zones. A thickening of the postsynaptic membrane can be observed at the tops of the folds. This thickened membrane region at the tops of the folds has been demonstrated to contain ACh-receptor (AChR) molecules located within the postsynaptic membrane (Gauthier, 1976). Filling the gap between the pre- and postsynaptic components is a fibrous meshwork known as the basal lamina. Associated with basal lamina are a large number of acetylcholinesterase (AChE) molecules (AChE is also present on the postsynaptic membrane). This enzyme, which splits ACh into choline and acetate, is partially responsible for removing transmitter from the cleft following its release.

Transmission at the neuromuscular junction occurs via release of ACh, a chemical transmitter, which diffuses across the synaptic cleft, and binds to AChR on the postsynaptic membrane. Upon excitation of a motor nerve and arrival of an

action potential at the nerve terminal there is an electrotonic depolarization of the nerve terminal. Depolarization of the terminal leads to the activation of voltage sensitive calcium-channels located within the terminal region. Ca^{++} flows into the nerve terminal and triggers (in some unknown way) the fusion of transmitter filled vesicles with the presynaptic membrane. Fusion of vesicles with the presynaptic membrane leads to the release of ACh into the synaptic cleft. ACh diffuses across the synaptic cleft and binds to AChR present in the postsynaptic membrane. Binding of ACh to its receptors leads to the opening of receptor-associated ion-channels. These channels open for several milliseconds and allow cations (primarily Na^{+} into the muscle cell and K^{+} out) to flow through them. At a normal resting membrane potential of approximately -90 mV, this predominantly inward cationic current leads to a depolarization of the end-plate region (Figure 2). The depolarization of the muscle cell membrane is known as an end-plate potential or EPP. Following nerve stimulation many ACh filled vesicles will fuse with the membrane and dump their contents into the synaptic cleft. The number of vesicles which are released per stimulation (or nerve impulse) is known as the quantal content (m) of the EPP, while a single transmitter filled vesicle is known as a single quantum of transmitter. It is possible to record an EPP by inserting a very fine microelectrode into the muscle cell membrane in the region of the end-plate. The amplitude of the EPP when measured in this way can range from a few millivolts to tens of millivolts. When recording from an end-plate region, random depolarizations are also observed which appear to be small EPPs.

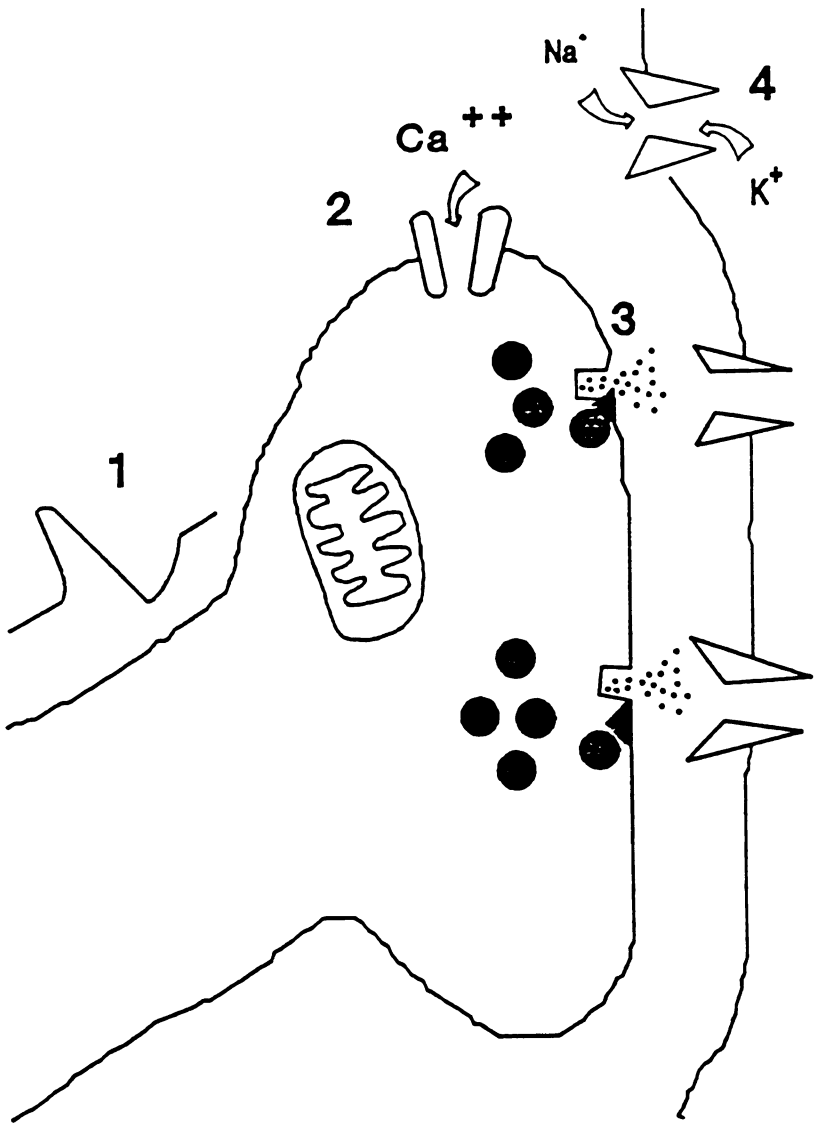


Figure 2. Neuromuscular Transmission.

Following stimulation of a nerve an action potential will be propagated down the axon and lead to a depolarization of the nerve terminal (1). Voltage sensitive Ca^{++} channels open and allow Ca^{++} to flow into the terminal (2). An increase in intraterminal Ca^{++} triggers the fusion of transmitter-filled vesicles with the presynaptic membrane and the subsequent release of transmitter into the synaptic cleft (3). Transmitter binds to receptor-channel complexes in the postsynaptic membrane increasing the permeability to Na^{+} and K^{+} (4).

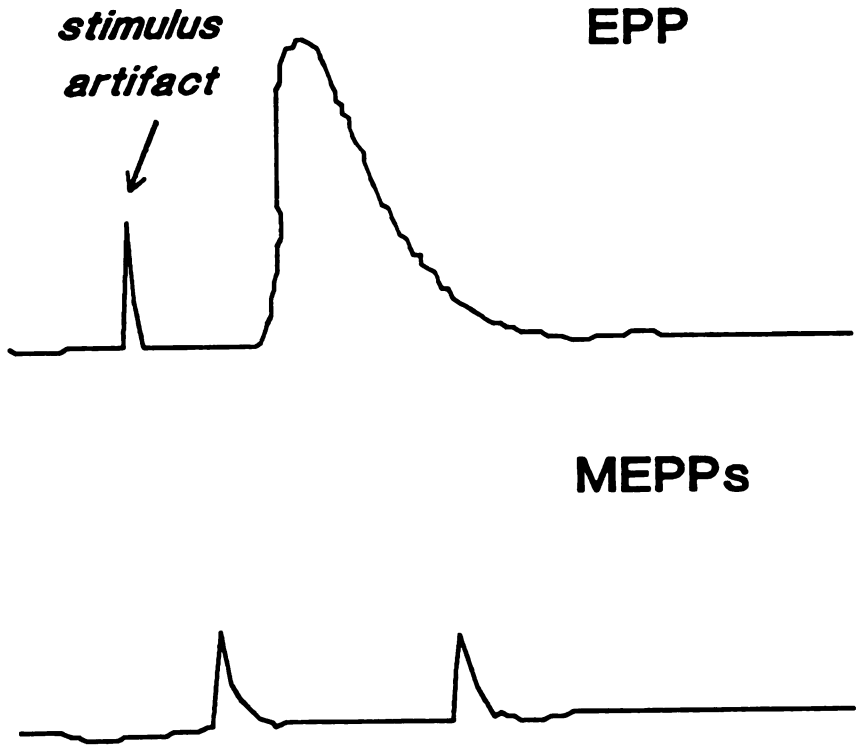


Figure 3. End-plate potential and miniature end-plate potential.

Following stimulation of the nerve, many synaptic vesicles will release their transmitter contents into the synaptic cleft leading to a large depolarization of the end-plate region. This depolarization of the end-plate region is known as an end-plate potential or EPP. The spontaneous release of the contents of a single synaptic vesicle (quantum) leads to a smaller depolarization known as a miniature end-plate potential or MEPP.

These are termed miniature end-plate potentials (MEPPs) and are believed to represent the spontaneous release of a single quantum of transmitter in the absence of nerve stimulation.

The nicotinic AChR to which ACh binds following its release is an integral membrane protein consisting of 5 subunits. These are 2 α subunits and one each of ß, γ or ϵ, and δ. The γ and ϵ subunits replace one another during different stages of development. The γ subunit is expressed in developing or denervated muscle, while the ϵ subunit is expressed in adult muscle (Mishina et al., 1986). The names of the subunits were chosen from the distance which they migrated on SDS polyacrylamide gels, with α having the highest mobility and δ the lowest (Steinbach and Ifune, 1989). These 5 subunits combine in such a manner as to form a pore through the membrane, which when open allows Na^+ into the cell and K^+ out. A variety of other positively charged molecules have been found to pass through the channel but do not appear to contribute to the current under physiological conditions. Not only do the subunits form a channel through the membrane, but they also contain the binding sites for ACh. These sites are located on the α subunits, thus each receptor-channel complex contains 2 binding sites. Also present on the α subunit is a reactive disulfide which is located very close to the binding site for ACh, reduction of which alters binding of ACh to the receptor (Ben-Haim et al., 1973).

Due to its high degree of accessibility and stability in vitro the neuromuscular junction has been well characterized both biochemically and electrophysiologically.

Many aspects of chemical transmission were first discovered using this preparation and have later been found to apply to other chemical synapses. Thus, the neuromuscular junction is often used as a model in which general aspects of transmission are studied or in which the effects of different pharmacological and toxicological treatments on synaptic transmission can be examined.

Chapter 2. Introduction to dithiobiuret.

Historical perspective. 2,4-Dithiobiuret (thioimidodicarbonic diamide, DTB) is a thiourea derivative with moderate reducing properties (Figure 4) (Preisler and Bateman, 1947). Compounds related to DTB and derivatives of DTB have been known since the early 1900s, however, the parent compound was not characterized fully until 1945 (Sperry, 1945). Following its characterization in 1945 a variety of studies investigated potential uses of DTB in both agriculture and industry. In agriculture DTB was proposed to be used as a promoter of seed germination and root growth (Dufrenoy and Pratt, 1948), as an insecticide or insect chemosterilant (Bousquet and Guy, 1945; Oliver et al., 1971; Chang et al., 1971), as a rodenticide (Dieke et al., 1947), or as a fruit thinner in peach orchards (Keil and Fogle, 1971). It was proposed to be used in industry as an intermediate in the production of resins and thermoplastics (Webb, 1952) or as a reducing agent in organic and analytical work (Preisler and Bateman, 1947). At present DTB is listed as being manufactured only for "specialty uses" by the chemical industry.

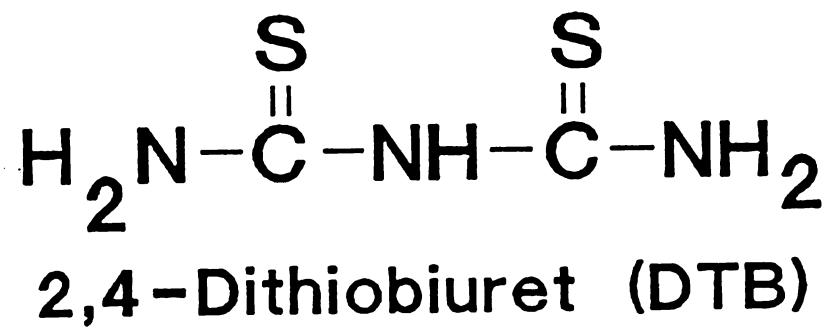


Figure 4. The structure of DTB.

Paralytic effects. In the early 1940's, while screening thiourea derivatives for antithyroid activity, Astwood (1943) observed that rats given small daily doses of DTB in their drinking water became paralyzed and died after approximately 1 week of treatment. Upon further characterization of the toxicity of DTB, Astwood et al. (1945) found that rats could be maintained in a paralyzed state for extended periods of time by decreasing the concentration of DTB in their drinking water at the time of paralysis, yet upon cessation of treatment paralyzed animals recovered apparently normal function in approximately 1 to 2 weeks. Results of this group's studies indicated that stimulation of motor axons in DTB-paralyzed rats caused contraction of the associated muscles. Thus they concluded that neither the motor nerve nor muscle were affected by DTB. Astwood et al. (1945) also reported an absence of morphological alterations in muscle, spinal cord or brain. Consequently, it was concluded that DTB most likely acted in the central nervous system on some poorly characterized aspect of transmission to cause the observed paralysis.

Following these early reports Altschul (1947) examined electroencephalograms of both dogs and cats treated with DTB in an attempt to characterize central effects observed in treated animals. In these studies no difference was observed in electroencephalograms recorded from control animals and those treated with DTB. An increase in the threshold for electrically-induced seizures was detected in rats severely paralyzed following treatment with DTB and the type of convulsion observed following electrical stimulation changed from a tonic-clonic type to a sustained tonic seizure. In light of these findings Altschul concluded, as did Astwood et al. (1945),

that DTB appeared to act centrally to cause the observed paresis.

In 1948 Seifter et al. published the results of a study in which skeletal muscle electrolyte levels and carbohydrate metabolism were examined in DTB-treated rabbits and rats. Their results demonstrated no change in the levels of skeletal muscle water, K^+, Na^+, Cl^-, phosphorus or creatine between control and treated animals. Seifter et al. did report increases in blood glucose following both acute and chronic exposure of rabbits to DTB. These could be antagonized by insulin or ergotamine. The conclusions resulting from these studies were that the paralysis observed in DTB treated animals was not due to an effect at the level of skeletal muscle and that the hyperglycemic effect was a nonspecific one mediated by catecholamines.

Additional reports on the mechanism of the paralytic effects of DTB were not published until the early 1980s when Atchison and Peterson (1981) characterized further and quantitated the time to onset and progressive nature of the DTB-induced weakness. Their results demonstrated that treatment of rats with DTB (1 mg/kg/day, ip) led to the loss in ability to walk on a rotating rod (rotarod test) after 4-6 days of treatment; loss of righting reflex after 6-9 days of treatment; and death following 6-10 days of treatment. It was observed that tail-flick, hindpaw withdrawal, corneal, pinna and vibrissae responses all could be elicited in rats exhibiting marked muscle weakness, indicating a lack of effect of DTB on sensory nerves mediating these reflexes. Treated rats also showed decreased intake of food and water, weight loss, a delayed onset diuresis, watery mucoid feces and chromodachryorrhea (a red

porphyrin secretion from around the eyes). Pair-fed control rats lost a comparable amount of weight to the treated rats, thus the decreased food intake and subsequent weight loss did not appear to be responsible for the weakness.

Atchison and Peterson (1981) also monitored rotarod performance in a group of rats which received a single 25 mg/kg dose of DTB, a dose which approaches the 48 hour LD_{50} in these animals (29 mg/kg). They found that this single large dose had no effect on rotarod performance when monitored in surviving rats for up to 3 weeks after treatment. Cholinesterase activity was also examined in rats treated with DTB to determine whether an alteration in its function was related to the observed weakness. Results of these studies demonstrated no change in whole blood cholinesterase activity.

Atchison and Peterson (1981) also reported an age and sex dependance for the effects of DTB. They found that females were more sensitive to the effects of DTB than males, while older rats of both sex were more sensitive than younger rats. Atchison et al. (1981a) discovered that altering the dose of DTB administered to rats altered the time of onset for rotarod failure. A dose of 0.125 mg/kg/day failed to cause muscle weakness after 52 days of treatment, while at doses ranging from 0.25 to 1 mg/kg/day weakness was observed following 5-20 days of treatment, depending on the dose. For this range of doses, it was found that a cumulative dose threshold of 4 to 5 mg/kg determined the onset of muscle weakness. When rats were given daily doses from 4-16 mg/kg/day the onset of muscle weakness became constant at 4 days.

Neuromuscular effects. The first evidence that a neuromuscular deficit was related to the observed weakness was reported by Atchison et al. (1981b). Experiments performed examined the tension of muscle twitches generated *in situ*, from muscles of control rats and rats exhibiting muscle weakness following treatment with DTB. In these experiments, twitches were elicited both by stimulation of the nerve (indirect) and by direct electrical stimulation of the muscle membrane. Results of these studies demonstrated that twitches in the gastrocnemius muscle elicited by indirect stimulation were decreased in treated rats compared to those measured in pair-fed controls. Tension developed following tetanic stimulation was also less in treated rats than controls, and muscles from DTB-treated rats displayed a marked fade in tension during stimulation. When twitch tension elicited by direct electrical stimulation of the muscle was monitored, no difference was noted between muscles of control or treated rats. These results indicated, for the first time, a possible impairment of axonal conduction and/or neuromuscular transmission in rats exhibiting muscle weakness following treatment with DTB. Further evidence for a prejunctional impairment in rats treated with DTB was observed by Atchison et al. (1982). They found in studies utilizing interrupted tetanic stimuli that rats exhibiting muscle weakness following treatment with DTB were somewhat resistant to the depression of contractile tension caused by hemicholinium-3, a blocker of high affinity choline uptake into the nerve terminal (Collier and MacIntosh, 1969; Guyenet et al., 1973). Concomitant treatment with choline, which normally antagonizes the hemicholinium-3 induced fatigue (Guyenet et al., 1973), worsened

the existing fatigue observed in rats treated with DTB. Atchison et al. (1982) reported that administration of 4-aminopyridine to rats exhibiting muscle weakness following treatment with DTB caused a return of twitch tension to control levels but did not overcome DTB-induced rotarod failure. 4-Aminopyridine blocks K^+ efflux through the so-called "delayed rectifier" potassium channels within the nerve terminal leading to an increase in the time that the terminal remains depolarized following nerve stimulation. This prolongation of terminal depolarization leads to an increase in the amount of transmitter that is released following nerve stimulation (Lundh, 1973; Pehlate and Pichon, 1974; Molgo et al., 1977). Thus, DTB treatment appears to be associated with a depression of transmitter release from motor nerve terminals that can be reversed by the actions of 4-aminopyridine. More evidence for an effect on choline metabolism has come from biochemical determinations of endogenous ACh and choline levels in rats treated with DTB. Results of these studies demonstrated an increase in both ACh and choline content in extensor digitorum longus muscles taken from rats treated with DTB compared to muscles of control rats. These findings indicate that DTB does not inhibit choline uptake or ACh synthesis. Instead it appears to disrupt processes responsible for ACh packaging or release (Weiler et al., 1986).

Electrophysiological findings. In light of the above results, several studies were performed which utilized intracellular recording techniques to characterize further the alterations in transmitter release detected in the above studies (Weiler et al., 1986; Atchison 1989). Results of electrophysiological experiments performed

on hindlimb muscles taken from animals treated with 1 mg/kg/day ip of DTB for 5 to 6 days have demonstrated a decrease in *m* of the evoked EPPs, a decrease in the frequency of spontaneously occurring and K^+-evoked MEPPs and an increase in the incidence of very large amplitude MEPPs with abnormal rise and decay times (Weiler et al., 1986; Atchison, 1989). An interesting finding reported by Atchison (1989) indicated that the decrease in *m* for EPPs could be partially reversed by increasing external Ca^{++} or by treatment with 4-aminopyridine, however, these manipulations were unable to restore *m* completely to control levels. This is in contrast to what was observed in earlier muscle twitch studies in which 4-aminopyridine treatment was able to restore twitch tension to control levels in muscles from DTB-paralyzed rats. One possible conclusion from these results is that DTB acts at a site after Ca^{++} has entered the terminal to cause some of the observed effects on quantal transmitter release.

Antagonism of DTB-induced muscle weakness. A characteristic of paresis produced by DTB treatment is that while small daily doses (0.25 to 5 mg/kg/day) reliably cause this weakness, large daily doses (above 12 mg/kg/day) do not cause muscle weakness. Williams et al. (1991) demonstrated that at a daily dose of 12 mg/kg/day flaccid neuromuscular weakness is not observed. These animals do display other signs of DTB intoxication such as watery mucoid feces, weight loss, chromodacryorrhea and a delayed onset diuresis (Atchison and Peterson, 1981; Williams et al., 1986). Inability to walk on a treadmill is observed in these rats following 3 to 4 days of treatment, but is not related to muscle weakness. Instead,

treated rats appear to be close to death and normally die within 24 hour of treadmill failure. These results are very interesting in that it appears as though DTB affords some protection against its own toxic effects when administered at high doses. Results of an earlier study by Williams et al. (1986) also demonstrated protection against DTB-induced muscle weakness by other sulfhydryl containing compounds. This study demonstrated that the sulfhydryl-containing compounds d-penicillamine, cysteamine, diethyldithiocarbamate and disulfiram all protected against the DTB-induced weakness. These compounds could both prevent DTB-induced muscle weakness if coadministered to animals with DTB and could reverse the DTB-induced weakness if given once weakness was observed. It was concluded that the protection against toxicity of DTB by sulfhydryl-containing compounds may indicate that some of DTBs effects are due to alterations of thiol/disulfide status within nerve cells.

Nonmotor alterations in peripheral transmission. Although motor function becomes severely impaired in DTB-treated rats, little evidence for an effect on sensory function has been noted. As described earlier, Atchison and Peterson (1981) observed no effect on tail flick latency, pinna, corneal and vibrissae reflexes in rats exhibiting marked muscle weakness following treatment with 1 mg/kg/day of DTB for 6 days. Crofton et al. (1991) performed an extensive series of tests on DTB-treated rats to determine whether DTB treatment altered sensory processing as well as motor function. They found that rats exhibiting muscle weakness following treatment with DTB demonstrated no change in thermal sensitivity, auditory function or pattern visual function, indicating that treatment with DTB does not

impair sensory systems. They did, however, report alteration in flash evoked potentials (FEPs) recorded with electrodes placed in the skull over the visual cortex. The FEPs which they recorded are thought to be generated within superficial layers of the visual cortex indicating an alteration in processing within the cortex itself, not a deficit in sensory input to the CNS (Crofton et al., 1991).

Pathological alterations in DTB induced weakness. A characteristic of many of the chemically-induced neuropathies including that by acrylamide (Kaplan and Murphy, 1972), cholinesterase inhibitors (Wecker et al., 1978) and the neurotoxic hexacarbons such 2,5-hexanedione (Spencer and Schaumburg, 1977) is the presence of high levels of neurodegeneration. Evidence for pathologic lesions in muscle or nerve of rats treated with DTB is lacking (Astwood et al., 1945; Atchison et al., 1981b). In a study performed at the light microscopic level, pathological alterations were not detected in liver, kidney, lung, thyroid, skeletal muscle, sciatic nerve, spinal chord or brain, even in rats maintained in a weakened state for extended periods of time (Williams et al., 1987). Kemplay (1984), using the zinc iodide-osmium staining technique, was able to detect small amounts of degeneration and nerve terminal remodelling in female rats treated with DTB. She observed both retraction and clumping of nerve terminals and an increase in terminal sprouting during the onset of muscle weakness. Axonal degeneration was detected in intramuscular nerve bundles within the sternocostalis muscle, while little or no degeneration was observed in nerves innervating the lumbrical, soleus and tibialis anterior muscles. Results of this study are also interesting in that in the past the

weakness had been described as ascending in nature (Atchison and Peterson, 1981), yet these findings indicate that some of the earliest and most striking changes were observed in the innervation to the sternocostalis muscles. This may indicate that the long axons to the lower extremities are not injured first, again differentiating DTB-induced muscle weakness from many other chemically-induced neuropathies. In a more recent study by Jones (1989), accumulations of branching smooth endoplasmic reticulum (SER) were noted within nerve terminals innervating the lumbrical muscles as early as 2 days following treatment of female rats with DTB. On days 3-5 of dosing, terminals became swollen and packed with organelles including large numbers of synaptic vesicles, dense core vesicles and SER. Evidence of nerve terminal sprouting was also observed at this time, as was terminal retraction and interposition of Schwann cells in the synaptic cleft. Jones also examined Ca^{++} levels within the terminals and muscle cells in control and treated rats. Results of these studies indicate a reduction in the intraterminal free Ca^{++} pool in nerve terminals of rats treated with DTB, detected as a decrease in oxalate-pyroantimonate precipitation of Ca^{++} within these terminals.

Summary. Rats treated with small daily doses of DTB begin showing signs of weakness following 2 days of treatment (Kemplay, 1984; Jones, 1989). By day 4-6 of treatment the weakness has progressed to the point where rats can no longer walk on a rotating rod or a continuously-moving treadmill (Atchison and Peterson, 1981; Williams et al., 1986). Morphological alterations, observed as an accumulation of smooth endoplasmic reticulum within nerve terminals are also

observed by the second day of treatment with DTB (Jones, 1989), while nerve terminal retraction and sprouting are observed by day 3 of treatment (Kemplay, 1984, Jones, 1989). Electrophysiological correlates to the observed weakness are a decrease in *m* of EPPs, a decrease in the frequency of potassium-evoked and spontaneous MEPPs and an increase in the frequency of failures of transmission following nerve stimulation (Atchison, 1989). The frequency of abnormally large slow MEPPs is also increased in muscles from rats exhibiting weakness (Atchison, 1989). There is some evidence that presynaptic alterations in the handling of Ca^{++} may play a role in the observed alterations in transmitter release. Jones (1989) observed a decrease in Ca^{++} content of terminals by 3-4 days of treatment with DTB. Atchison et al. (1982) observed that administration of 4-aminopyridine, to rats exhibiting muscle weakness following treatment with DTB, caused a return of twitch tension to control levels. Exposure to 4-aminopyridine or increasing extracellular Ca^{++} did not completely restore quantal content, which was depressed in muscles from treated rats, to control levels nor did it cause recovery of normal motor function. Thus, the observed depression in transmitter release does not appear to be due solely to alterations in presynaptic Ca^{++} handling.

Chapter 3. Significance of toxicity of DTB.

Neuromuscular junction as a model synapse. As described in Chapter 1 the neuromuscular junction is often used as a model in which to study synaptic transmission in general. Many aspects of chemical transmission which are understood at this time were first studied at this synapse. By understanding how a chemical like DTB disrupts transmission, the maintenance of normal synaptic contacts and the disruption of transmission during other compromised states may be better understood.

Novel type of muscle weakness. When examined closely the actions of DTB and the neuromuscular weakness it causes appear very unique when compared to those of well characterized agents known to cause weakness or paralysis. Neuromuscular blocking agents such as d-tubocurarine, pancuronium and succinylcholine, although they act by different mechanisms, block transmission soon after administration of a single dose (Miller, 1984). This is in contrast to the actions of DTB for which the observed weakness is determined both by a minimum time of exposure and cumulative dose threshold (Atchison et al., 1981a). Other agents have been characterized which also require repetitive exposure over prolonged times before their effects are observed. Compounds such as the neurotoxic hexacarbons (n-hexane, 2,5-hexanedione) and acrylamide require time after initiation of exposure before paralysis or weakness is observed (Kaplan and Murphy, 1972; Spencer and Schaumburg, 1977). Studies examining pathological alterations during the onset of

paralysis with these agents in all cases reveal large amounts of neurodegeneration (Kaplan and Murphy, 1972; Spencer and Schaumburg, 1977). In light of this, the progression of the changes observed with these agents has been classified as a "dying back" or "distal axonopathy" (Spencer and Schaumburg, 1977). By this it is meant that long fibers innervating muscles of lower extremities are affected first, while effects spread to more proximal nerves with continued exposure. Upon cessation of treatment or exposure, affected animals do recover normal neuromuscular function. However, it can take weeks to months for this recovery to occur. This is not the case for DTB. Very little neurodegeneration is observed in rats treated with DTB (Kemplay, 1984; Jones, 1989). The neurotoxic hexacarbons and acrylamide affect both motor and sensory nerves (Kaplan and Murphy, 1972; Spencer and Schaumburg, 1977), while in DTB-induced muscle weakness sensory systems appear to be spared (Atchison and Peterson, 1981; Crofton et al., 1991). Thus, DTB appears to have actions which are in many ways different from those of classical blockers of neuromuscular transmission or other neurotoxic agents which cause paralysis.

Comparison with human neuromuscular disorders. Similarities exist between electrophysiological characteristics of weakness observed in rats treated with DTB and those observed in patients suffering from several neuromuscular diseases. Electrophysiological analysis of muscle biopsies from patients suffering from one myasthenic disorder demonstrated decreased MEPP frequency and quantal content of evoked EPPs and increased duration of MEPP half-decay time (Engle et al.,

1977). Results from studies of patients suffering from another myasthenic disorder, congenital myasthenic syndrome, which is proposed to be due to prolongation of the open-times for ACh channels at the neuromuscular junction, showed an increase in the duration and half-decay time for EPPs, MEPPs and miniature end-plate currents (MEPCs), a decrease in MEPP amplitude and normal quantal content (Engle et al., 1980; 1982). Following exposure to botulinum toxin (from Clostridium botulinum) EPP amplitude is decreased, the incidence of failures in transmission is increased and frequency of MEPPs decreased. Thus, neuromuscular weakness induced in rats by DTB may be useful as a model system in which to study neuromuscular deficits associated with several diverse neuromuscular diseases in humans.

Chapter 4. Materials and methods.

During the course of the experiments described herein three different electrophysiological recording techniques were utilized. These different techniques allowed for the acquisition of information regarding the action of DTB on AChR-channels which could not have been obtained using any one of the techniques alone. In the following section is a description of the methods used in gathering data using single electrode current clamp, patch voltage clamp, two microelectrode voltage clamp and fluctuation analysis using two microelectrode voltage clamp in conjunction with iontophoresis of ACh. Also included are descriptions of the preparations studied using the above techniques.

Preparations and Treatments.

Intact neuromuscular preparations. All experiments utilizing single electrode current-clamp and two microelectrode voltage-clamp were performed using the isolated phrenic nerve-hemidiaphragm preparation (Bülbring, 1946) of male rats (180-250g, Harlan Sprague-Dawley Laboratories, Madison, WI). This muscle was chosen because it is a flat, relatively thin preparation, and can be maintained for long periods of time in vitro. It was important to have a preparation which was only a few fibers thick so that terminal regions could be readily observed and penetrated during two-microelectrode voltage clamp and iontophoretic experiments. In many

of the studies in which DTB was added to the bathing medium, preparations were observed for several hours. Thus the nerve and muscle must be stable for several hours in vitro. Hemidiaphragms, with associated phrenic nerves, were dissected from control rats and rats treated previously with DTB. Muscles fibers were cut approximately 4 mm on either side of the intramuscular nerve branch to prevent contractions due to stimulation of the phrenic nerve (Barstad and Lilleheil, 1968; Hubbard and Wilson, 1973; Glavinovic, 1979; Traxinger and Atchison, 1987a). Cutting the muscle in this way causes depolarization of the muscle cell membrane, yet does not alter cable properties of the muscle or transmitter release (Glavinovic, 1979; Fiekers, 1983; Atchison, 1986). Following transection, muscles were incubated for 1 hour in ice cold, low K^+ buffer and then transferred to a Sylgard-coated, Plexiglas chamber and pinned out and allowed to equilibrate for approximately 30 minutes prior to recording to restore normal ionic gradients disturbed by incubation at low temperatures (Traxinger and Atchison, 1987a,b). The use of buffer with a lower K^+ concentration (2.5 mM vs 5 mM in normal extracellular buffer) prevents K^+, which leaks out of the transected muscle fibers, from causing conduction block of nerves innervating the muscle. During recording sessions the diaphragm was superfused continuously with a low K^+ physiological saline solution modified from that described by Liley (1956); it contained (mM): NaCl, 137.5; KCl, 2.5; $MgCl_2$, 1; $CaCl_2$, 2; d-glucose, 11; and N-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), 14. Solutions were continuously oxygenated with 100% O_2. All experiments were performed at room

temperature and in buffers of pH 7.4.

Rats treated chronically with DTB. Chronically-treated rats were given 1 mg/kg/day ip of DTB dissolved in 0.9% NaCl (1 mg/ml) for 6 to 8 days. Rats treated in this manner develop muscle weakness after approximately 4-6 days of treatment and normally fail the rotarod test for 2 consecutive days by 6-8 days of treatment (Atchison and Peterson, 1981; Atchison *et al.*, 1981a,b). Previous experiments used 2 days of rotarod failure as an index to insure that all rats had attained the same level of weakness prior to their use in electrophysiological recording experiments (Atchison, 1989).

Rats treated with a single large dose of DTB. To determine the time course for the onset of effects of DTB on neuromuscular transmission rats were treated with a single 25 mg/kg (ip) dose of DTB (dissolved in tween-80: ethanol (95%): and NaCl (0.9%), 1: 1: 8, v/v/v to a final concentration of 5 mg/ml). Rats were sacrificed and diaphragms removed 1, 4, 8 and 24 hour after treatment. Although this dose approaches the 48 hour LD_{50} in rats, it has been shown to cause no observable muscle weakness in rats monitored for up to 30 days following treatment (Atchison and Peterson, 1981). By using a maximally tolerated dose, which does not cause muscle weakness, we hoped to observe very early alterations in neuromuscular transmission which may progress to the weakness observed in chronically treated rats.

Rats treated with a single low dose of DTB. Several of the fluctuation analysis recordings were performed in muscles taken from rats 24 hour following treatment with a single 1 mg/kg dose of DTB. From these experiments we hoped to determine

whether effects observed in muscles taken from rats exhibiting muscle weakness following 6-8 days of treatment with 1 mg/kg/day could be detected following a single 1 mg/kg dose.

Bath exposure of control muscles to DTB. The earliest times at which the effects of DTB could be examined in hemidiaphragm preparations from rats treated with DTB were approximately 2-2.5 hr following initial exposure to DTB. Most of this time was required for incubation in cold saline followed by equilibration at room temperature. To determine whether DTB affected transmission at earlier times than could be measured in muscles from treated rats, diaphragms were removed from untreated rats and exposed to DTB in the bathing medium. Diaphragms were removed from control rats and handled in the same manner as described above. After equilibration to room temperature, control MEPPs and EPPs (1.0 Hz stimulation frequency) were recorded for 5 minutes each, after which time the perfusion solution was changed to one containing DTB. In the initial current clamp studies two concentrations of DTB were used: the high concentration of 1.85 mM, was calculated to be the plasma concentration if DTB were to distribute to 10 % of body weight (plasma alone) and the low value of 200 μM was calculated to be the plasma concentration if DTB were to distribute to 70 % of body weight (whole body water), 1 hour following a single dose of 25 mg/kg DTB. Porter et al. (1983) reported a plasma DTB concentration of 14.04 μg/g (approximately 150 μM) 3 hour following a single 25 mg/kg dose of DTB, which supports our calculated values for 1 hour after the 25 mg/kg dose. In fluctuation analysis experiments a value of 1 μM

DTB was used. This concentration was chosen for two reasons. First, previous patch voltage clamp experiments had demonstrated an effect on single channel open-time following exposure to this concentration of DTB. Second, treated rats used in fluctuation analysis studies received daily doses of 1 mg/kg/day, a dose level which results in a plasma concentration of DTB derived equivalents of approximately 1 μM 24 hour following treatment (Porter et al., 1983).

Cultured G8 muscle cells. A requirement for patch voltage clamp is direct access to a relatively clean membrane surface (Hamill et al., 1981). This is seldom, if ever, attained in intact mammalian neuromuscular preparations. Due to these limitations all patch voltage clamp studies were performed on a clonal cell line. The cell line which was used was a mouse myoblast cell line (G8 passage 20-30). These cells express nicotinic AChR and have been well characterized electrophysiologically (Morris and Montpetit, 1986; Morris et al., 1989). A muscle cell line was chosen over other cell lines which express nicotinic AChR since future studies plan to use cocultures of nerve and muscle cells to study effects of DTB on synaptic activity within these cultures. G8 cells were grown in collagen-coated 30 mm culture dishes. Cells were grown in Dulbecco's modified eagles medium containing 10% fetal bovine serum and 10% horse serum. Differentiation of myoblasts was enhanced by growing cells in normal growth medium until confluence, then decreasing the serum content to 2% fetal bovine serum and 2% horse serum. Following a decrease in the serum content, these cells differentiate from small, mononucleated, spindle shaped cells to form multinucleated myotubes, which are highly responsive to ACh (Morris et al.,

1989). Electrophysiological recordings were performed on differentiated myotubes within 3-4 days of differentiation.

Exposure of cells in culture to DTB. Two protocols for exposure of cells to DTB were used 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 μM ACh or 100 nM suberyldicholine (SCh_2) followed by exposure to agonist plus DTB (1-1000 μM). In the second group of experiments, cells were grown in growth medium containing DTB (1-10 μM). Cells died when grown in medium containing DTB at concentrations of 100 μM or more. Following exposure of cells to DTB for 1-3 days single-channel currents were recorded in the presence of 0.1-2 μM ACh.

Electrophysiological Recording Procedures.

Single microelectrode current clamp studies. Synaptic potentials were recorded using borosilicate glass microelectrodes (15-25 MΩ impedance) filled with 3 M KCl (Figure 5). Synaptic potentials were amplified and subsequently displayed on a digital oscilloscope (Model 2040, Nicolet Instruments, Verona, WI) and recorded on magnetic tape using an FM instrumentation tape recorder (Model B, Vetter Instruments, Rebersburg, PA). EPPs were elicited via stimulation (supramaximal constant current, 70 microsecond duration) of the phrenic nerve with a suction electrode and a 1830 interval generator, 1831 pulse train module, and a 1850A stimulus isolator (WPI Instruments, New Haven, CT).

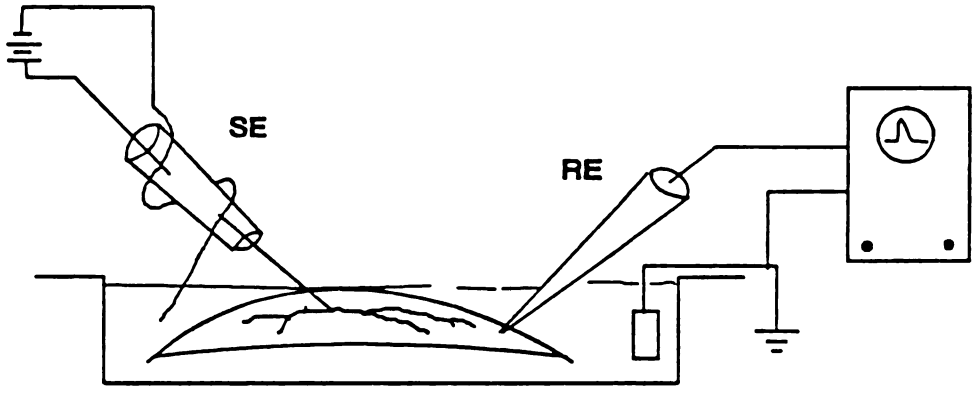


Figure 5. Setup for recording under current clamp.

Hemidiaphragm preparations were removed from control and treated rats and transected to prevent contraction following stimulation of the phrenic nerve. Muscles were pinned down in a Sylgard-coated Plexiglas chamber and perfused with physiological saline. End-plate regions were impaled with a KCl-filled microelectrode and EPPs and MEPPs recorded.

EPP amplitude, MEPP amplitude and frequency and rise and decay rates for both MEPPs and EPPs were analyzed using the analysis program pClamp® (Axon Instruments, Foster City, CA) on a Zenith microcomputer (Model ZW-248-84). Rise time was measured as the time elapsed from the start of rise of an event to the peak of the event, while decay time was measured as the time from peak amplitude to the resting potential. EPP amplitudes were corrected for nonlinear summation (MacLachlan and Martin, 1981; Traxinger and Atchison, 1987a) and standardized to a membrane potential of -50 mV (Katz and Thesleff, 1957) as described by Atchison (1989). Quantal content was calculated by dividing the mean EPP amplitude by the mean MEPP amplitude (mean value method, (Del Castillo and Katz 1954; Hubbard et al., 1969)). Typically 1-3 end-plate regions were recorded from in each preparation. Impalement was considered acceptable if rise times for MEPPs were approximately 1 millisecond, while rise times for EPPs were approximately 1.5 milliseconds. At each end-plate region, MEPPs were recorded continuously for 5 min. Following this, EPPs were elicited by electrical stimulation of the phrenic nerve at frequencies of 0.5, 2, 5, 25 and 50 Hz to determine whether there was any frequency-dependance to the effects of DTB. Two minutes were allowed to pass between the first three stimulation frequencies and 5 minutes between the next two for redistribution of Ca^{++} following stimulation. For statistical purposes at least 200 EPPs were recorded at each stimulus frequency.

In muscles which were exposed to DTB in the bathing medium MEPPs were recorded for 5 minutes and EPPs (1.0 Hz) for 4 minutes prior to DTB exposure.

After recording control potentials the perfusing solution was changed to one containing DTB (200 μM or 1.85 mM) and EPPs (1.0 Hz) were recorded continuously until block of EPP was observed. In preparations in which block did not occur within 30 minutes, MEPPs were counted for 5 minutes and at 10 minute intervals thereafter until block occurred or until the preparation was exposed to DTB for 60 minutes. After 60 minutes of exposure to DTB, the preparation was washed with a DTB-free control solution and monitored while stimulating at 1.0 Hz for approximately 30 minutes. In preparations in which block of the EPP occurred within 30 minutes, stimulation of the phrenic nerve was halted, MEPPs were recorded for 5 minutes, and then the preparation was washed with a DTB-free buffer and nerve stimulation resumed until EPPs returned.

Two microelectrode voltage clamp recordings. Voltage clamp experiments were conducted using the two microelectrode voltage clamp technique (Takeuchi and Takeuchi, 1959) and hemidiaphragm preparations prepared as described above (Figure 6). Voltage recording and current passing electrodes had impedances of 5-10 and 2-4 MΩ respectively when filled with 3 M KCl. Lower resistance microelectrodes were used for current passage; this allowed the passage of large amounts of current necessary to maintain space-clamp of the end-plate region of the muscle cells. With the two microelectrodes in the bath, the series resistance of electrodes can be neutralized via the bridge balancing circuitry of the amplifier. This also allows measurement of the electrode resistance directly from the adjustment dial on the amplifier.

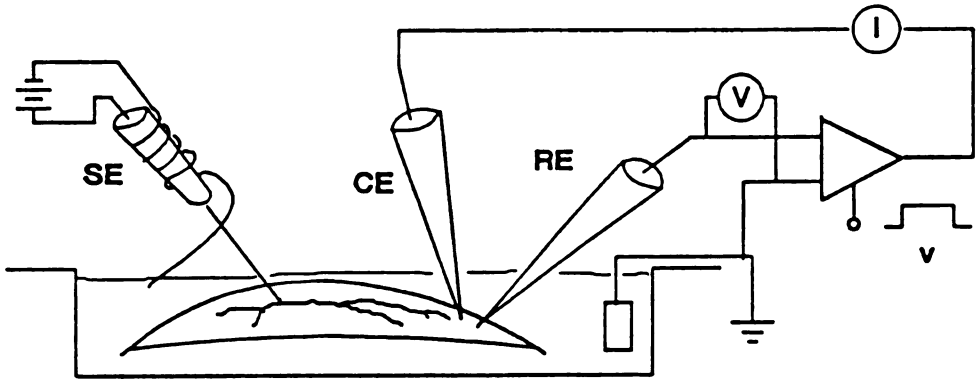


Figure 6. Setup for recording under voltage clamp.

Hemidiaphragm preparations were prepared as described in Figure 5. End-plate regions were impaled with two microelectrodes. One electrode monitors membrane potential via a voltage follower circuit. During a change in membrane potential, which is sensed by the voltage follower, current is passed through the second electrode to return the membrane potential to its original level. Using this configuration the membrane potential can be held (clamped) at a constant level and currents can be recorded which underlie the synaptic potentials described above.

End-plate regions were located and impaled with the low resistance current passing electrode, followed by the high resistance voltage recording electrode. Impalement in this order was more successful due to the fact that the current recording electrode has a finer tip and tends to disturb an already impaled cell to a lesser degree than impaling in the other order. Locating the same cell with both electrodes is done by passing a small amount of current through the second electrode (the voltage recording electrode in this case) and penetrating cells in the region until a step depolarization is observed in the electrode already within the cell (the current passing electrode in this case). When both electrodes are located within the same cell a step change in voltage can be observed in one electrode following current injection from the other electrode and MEPPs with similar rise and decay times should be observed simultaneously through both electrodes (Figure 7). Following impalement of a single cell with both electrodes, the capacitive coupling between the two electrodes can be minimized using the capacitive compensation circuitry on the amplifier. Capacitive coupling between the electrodes can also be minimized by maintaining the bath level as close to the surface of the muscle as possible. Voltage clamp steps were made from a holding potential of -50 mV using the Axoclamp-2 voltage clamp circuit (Axon Instruments Inc., Foster City, CA). EPCs were elicited by nerve stimuli (1 Hz, 70 microsecond duration) applied through a suction electrode with a Grass S88 stimulator and a SIU5 stimulus isolation unit (Grass Instruments, Quincy, MA).

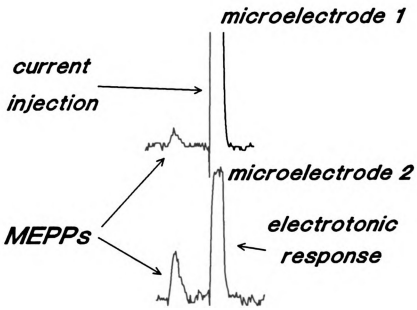


Figure 7. End-plate penetrated with two microelectrodes.

Upon successful penetration of an end-plate region with a microelectrode MEPPs can be observed which have rise times of 1 millisecond or less. By placing a second electrode in the same cell MEPPs can be observed simultaneously through both electrodes and current injected through one electrode can be detected with the second electrode.

At each holding potential 20-30 EPCs and 100-200 MEPCs were recorded on FM tape with a 0-2.5 kHz bandwidth (Store 4DS, Racal Recorders Inc., Irvine, CA) and analyzed off-line using a Zenith microcomputer with a maximum sampling rate of 83 kHz (model ZW-248-84). EPCs and MEPCs were digitized and the decay time constant (τ) was obtained from a curve fitting routine which fit an exponential curve to the falling phase of EPCs or MEPCs (pClamp®, Axon Instruments, Foster City, CA). Analysis of modal amplitude MEPCs was accomplished by creating an average MEPC from all MEPCs recorded at a single end-plate region which had amplitudes equal to the mode value for that end-plate. These MEPCs were selected first by amplitude and then sorted manually to eliminate MEPCs with prolonged rise and decay times. The edited MEPCs were then summed to give an average modal MEPC, and amplitude, 10-90% rise time and τ were calculated. τ_{MEPC} for the modal MEPCs was obtained from the inverse of the slope of semilog plots of the decay phase of the summed MEPCs (Fiekers, 1981). Separate Gaussian distributions were fitted to the MEPC amplitude histograms using the pSTAT portion of pClamp® (Axon Instruments, Foster City, CA). Quantal content was calculated by the mean value method (Del Castillo and Katz 1954; Hubbard et al., 1969).

Patch voltage clamp. 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) (Figure 8).

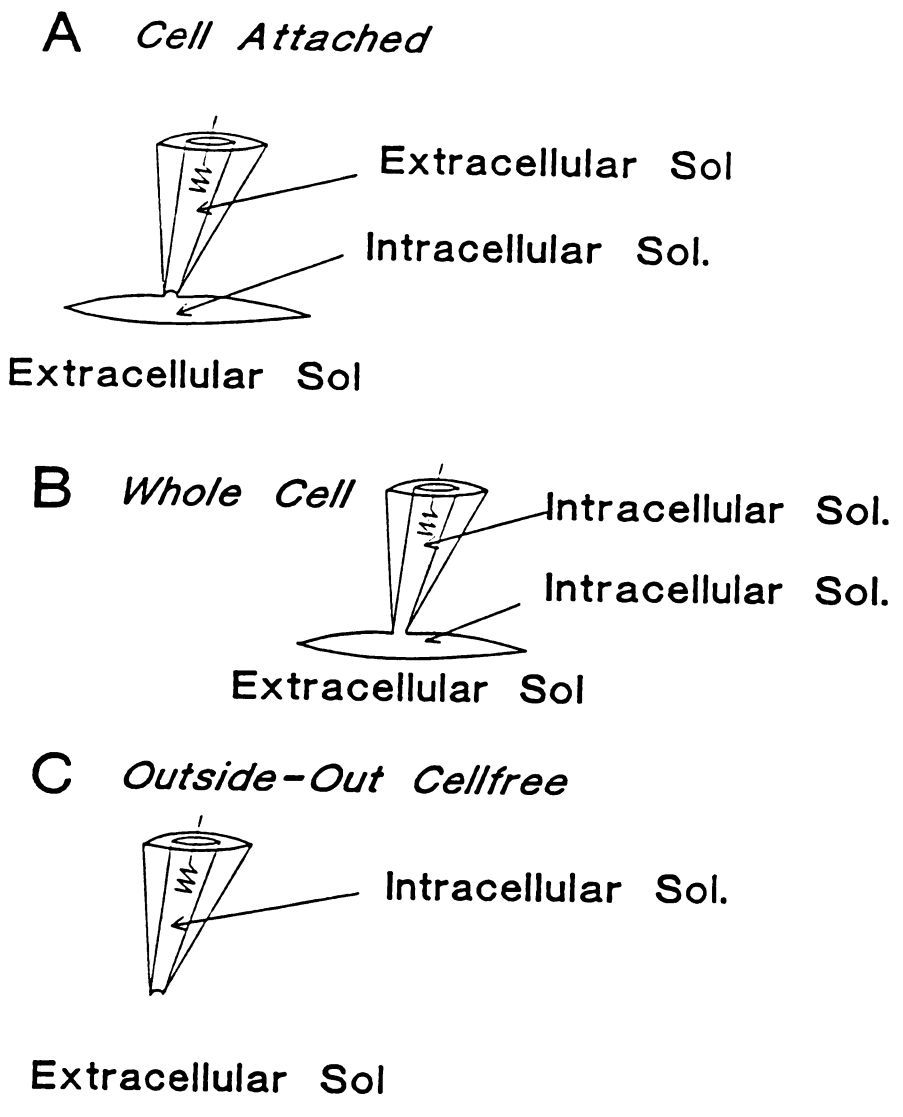


Figure 8. Setup for recording under patch voltage clamp.

Formation of a high resistance seal (1-100 gigaohm) between a fire polished pipet and a cell membrane can be accomplished by touching the pipet to the membrane and applying negative pressure to the interior of the pipet. This procedure isolates a small patch of membrane from the rest of the cell membrane, and is referred to as a cell-attached patch (A). Additional application of negative pressure ruptures the patch of membrane spanning the mouth of the pipet, which allows both electrical and physical access to the interior of the cell (whole-cell configuration (B)). Withdrawal of the pipet from the cell leads to the formation of a cell-free, outside-out patch spanning the tip of the pipet (C).

During electrophysiological recording the bathing medium was changed to a physiological saline; it contained (mM) NaCl, 135; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; *d*-glucose, 11 and HEPES, 14 (set to pH 7.3 with NaOH). Patch electrodes (5-10 MΩ resistance) were filled with saline containing; (mM); KCl, 135; $MgCl_2$, 2; *d*-glucose, 10; HEPES, 10; ethylene glycol-bis-(ß-amino ethyl ether) N,N-tetraacetic acid (EGTA), 2 (set to pH 7.3 with KOH). All solutions used were filtered prior to use to remove small contaminating particles which could impede seal formation (Whatman #1 filters). Prior to patch formation, positive pressure was maintained on the inside of the patch electrode (pressure adjustments were made with a 1 cc syringe attached to the inlet port of the electrode holder) to prevent cell debris in the perfusing solution from sticking to the end of the microelectrode. During seal formation the microelectrode was brought into contact with the membrane of a differentiated myotube. The point of contact can be determined visually by observing an indentation of the membrane surface at the tip of the pipet and by a change in the audio response of the patch clamp circuit during repetitive voltage pulses. In addition, the amplitude of the current observed during repetitive voltage pulses will decrease as the microelectrode forms a seal with the membrane (the resistance of this initial seal is on the order of 50-100 MΩ). Following initial seal formation negative pressure was applied to the interior of the microelectrode; this normally leads to the formation of a very high resistance seal on the order of 1-100 GΩ (giga-seal). Upon formation of a "giga-seal" the current step observed during a pulse of voltage across the membrane will be decreased to a very low level, leaving only

capacitive transients at the start and finish of a step change in voltage change. If formation of a high resistance seal does not follow the application of negative pressure to the interior of the microelectrode, imposition of a moderate hyperpolarization across the patch (10-50 mV) often aided in its formation. Following formation of a high resistance seal between the microelectrode and cell membrane, the patch of membrane spanning the interior of the microelectrode can be ruptured to form what is known as the "whole-cell" configuration. The membrane patch can be ruptured by increasing the negative pressure within the microelectrode further, by passing an oscillating voltage across the membrane patch or a combination of these two methods. Following rupture of the membrane patch within the microelectrode, the capacitive current changes observed at the start and finish of a current pulse were increased in amplitude and duration, indicating an increase in membrane area from which capacitive current is measured. To obtain a detached patch from the whole-cell configuration the microelectrode was slowly withdrawn from the membrane surface. Upon withdrawal of the microelectrode from the surface of the cell, a small patch of membrane formed across the tip of the microelectrode. If the microelectrode was pulled away from the cell surface when in the whole-cell configuration, the patch of membrane at the tip of the microelectrode had its extracellular face on the outside of the microelectrode ("outside-out cell-free patch") (Hamill et al., 1981). The resistance of the cell-free patch could sometimes be improved by imposing small amounts of positive or negative pressure to the inside of the microelectrode. Once a high resistance seal

was obtained in a cell-free patch, pressure on the inside of the microelectrode (either positive or negative) should be removed to prevent the opening of pressure sensitive channels. Single-channel currents were amplified 100X 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). Single-channel currents were recorded to two different channels on the tape. One channel was filtered using a low-pass filter (Bessel response) with a cutoff frequency of 2 kHz. The second channel was not filtered prior to recording to allow later analysis in the absence of filtering, or following filtering at different frequencies. During analysis, current recordings were sampled at 50 microsecond intervals, stored on hard-disc and analyzed off-line using the pClamp® analysis software (Axon Instruments, Foster City, CA) on a Zenith microcomputer (model ZW-248-84). The opening and closing of channels was detected by a half-amplitude current threshold (Colquhoun and Sigworth, 1985). Due to the limited bandwidth of the data analysis system, the minimum duration of event which was detectable was 100 microseconds. This was determined by measuring the minimum duration of a voltage step (generated by a Grass S88 stimulator) which could be accurately measured using the acquisition system. 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. 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 vs voltage relationship for a given amplitude class of channels. In most patches multiple conductance levels were observed, however, channel openings with a slope conductance of 37 pS predominated. Only this conductance class was used in further analysis.

Fluctuation Analysis. The membrane potential of end-plate regions of muscles from control rats and rats treated with DTB was clamped using the two microelectrode voltage clamp techniques described earlier. Following successful clamp of the membrane potential at an end-plate region, a third microelectrode (iontophoretic electrode) containing 2 M ACh was brought into the region of the end-plate (Figure 9). The ACh containing electrode was connected to a WPI intracellular recording amplifier. Using this amplifier allowed the resistance of the electrode to be monitored (normally 50-100 MΩ) and for the passage of current (depolarizing and hyperpolarizing). When positive current is passed through the iontophoretic microelectrode, it will force ACh out of the electrode (due to ACh's positive charge) into the region of the end-plate. To prevent ACh from leaking from the electrode when not iontophoresing, a negative retaining current was passed (2-4 pA). By placing the iontophoretic electrode very close to the end-plate region (actually into the connective tissue), very fast currents could be elicited by iontophoresis of ACh into the region. However, placement of the iontophoretic electrode in this manner normally leads to the displacement of one or both of the voltage clamp microelectrodes from the end-plate region.

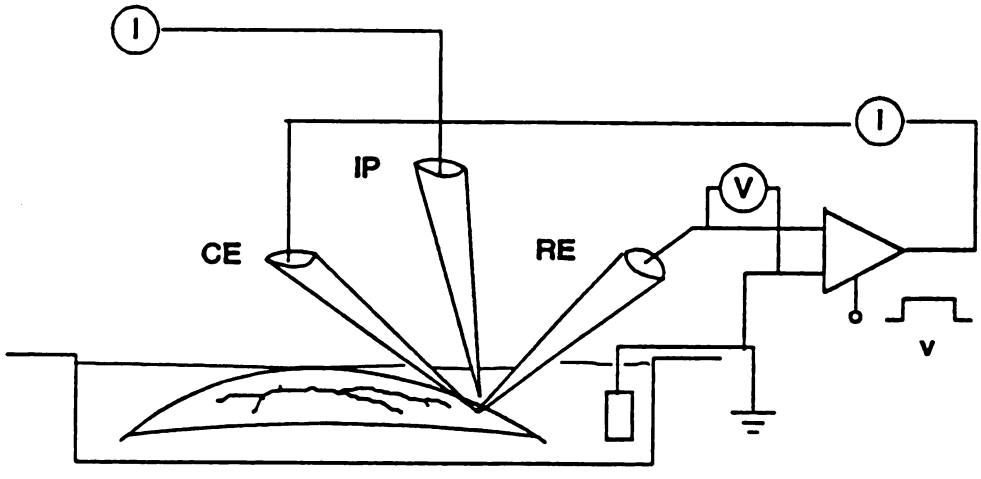


Figure 9. Setup for recording ACh-induced membrane noise.

The membrane potential of end-plate regions were held constant using two microelectrode voltage clamp as described in Figure 6. A third ACh-containing microelectrode was brought into the region of the end-plate. By passing a positive current through the ACh-containing electrode, ACh is ejected into the region of the end-plate. Steady application of low levels of ACh to the end-plate region causes membrane noise to increase due to the random opening and closing of a number of AChR-gated ion channels. Information regarding the conductance and open-time of these channels can be determined from the amplitude and frequency of the observed fluctuations.

Better results for the recording of membrane noise were obtained by placing the iontophoretic electrode a short distance from the end-plate region. By passing low levels of positive current (2-10 pA) from an iontophoretic electrode placed near the end-plate region, EPCs on the order of 2-20 nA could be elicited which could be maintained for many seconds (up to 60 seconds). Membrane noise, in the absence and presence of ACh, was recorded at 1-4 different end-plate regions in each hemidiaphragm preparation at holding potentials from -20 to -70 mV. Noise containing signals before and after iontophoresis of ACh were recorded to FM tape (15 ips, Store 4DS, Racal Recorders Inc., Irvine, CA) for storage prior to analysis. During analysis, noise-containing signals were sampled at 50 microsecond intervals, stored on hard-disc and analyzed off-line using the program SPAN (Spectral Analysis Program, J. Dempster, University of Strathclyde, Glasgow, G1) on a Compac microcomputer. For analysis two channels of noise-containing signal were recorded to computer. One channel was DC-coupled and amplified 1 to 10X. This channel was used to determine the amplitude of the iontophoretic current. The second channel was AC-coupled and was amplified 100-1000X. 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 (VBF/8 dual channel variable filter, Kemo Limited, Kent, UK). Power spectra for the recorded noise was calculated, one or two Lorentzian curves were fit to the calculated spectra and the decay time constant for channels were determined from

the corner frequency ($f_{(c)}$) of the calculated spectra (all were done using the program SPAN). Channel conductance could be determined from amplitude of the macroscopic current and the standard deviation of the ACh induced noise or from the maximum power level of the calculated spectra (SPAN).

Chemicals. Purified, recrystallized DTB was obtained from Ash Stevens (Detroit, MI). HEPES was obtained from United States Biochemical Corporation (Cleveland, Ohio). ACh and SCh_2 were obtained from the Sigma Chemicals (Milwaukee, WI).

Statistics. Slopes of calculated regression lines were compared using a t-test for the comparison of slopes. Specific points on these lines were compared using a t-test for comparing the height of points on separate lines, and the means of treatment group values were compared to control values using an Analysis of variance, comparisons between groups were made using Dunnet's t-test and the means before and after exposure to DTB were compared using a Student's t-test (Steel and Torrie, 1980). Significance was set at $p < 0.05$.

Chapter 5. Effects of acute exposure to DTB on neuromuscular transmission.

Purpose of studies. One possible explanation for the cumulative dose threshold and latency to onset of paresis is that it takes time for DTB or a metabolite thereof to accumulate in the rat prior to manifestation of muscle weakness. This is quite possible when one considers that the plasma half-life of DTB or DTB-derived equivalents is between 8 and 9 hour in rats (Williams et al., 1982; Porter et al., 1983). Thus only 88% of the DTB given in a daily dose would be excreted by the time the next dose was administered. Two sequences of events may be observed during accumulation of DTB. First, as DTB, or a metabolite thereof, begins to accumulate subtle changes in transmission may occur. With continued exposure to DTB these alterations increase in magnitude as the level of the toxic compound increases within the organism. Conversely, transmission may not be affected until DTB or a metabolite thereof reaches a toxic level, at which time transmission is affected and paralysis observed. Accumulation of DTB or a metabolite may not be critical; instead some process critical to transmission may be inhibited by DTB. Then continuous exposure to DTB would lead to continuous interference with the critical process, slowly decreasing transmission over the course of treatment. That is, DTB treatment may produce a subtle gradual disruption of synaptic transmission which only becomes evident as frank paralysis after 3-6 days of continuous dysfunction. Therefore, the purpose of the studies described below was to determine whether subtle changes in neuromuscular transmission could be

detected at early times following a single large dose of DTB when no muscle weakness is observed in the whole animal, or at very early times following exposure to DTB using a perfusion system and isolated tissue. If so, these effects might progress to a more generalized weakness with time and continued exposure to DTB. Conversely, it may take several days of treatment before DTB, or a metabolite thereof, reaches a level at which transmission is affected. If this is true then no effects should be observed following acute treatment.

Results.

Effects of DTB on animal appearance and behavior. At 1 and 4 hour following a single injection of 25 mg/kg of DTB, rats appeared normal, while at 8 and 24 hour after treatment they appeared lethargic. Treated animals could move when disturbed, however their normal exploratory behavior appeared to be suppressed. Several other signs of DTB intoxication such as diarrhea and lack of grooming were also observed in these rats. In one rat in which electrophysiological experiments were not performed the above signs of intoxication were observed up to 24 hour; by 48 hour this rat appeared normal.

Electrophysiological studies on muscles from rats treated with DTB. Results from electrophysiological studies demonstrate that increasing stimulus frequency, from 0.5 to 50 Hz, caused a steady decline in EPP amplitude in muscles taken from both control and treated rats (Figure 10).

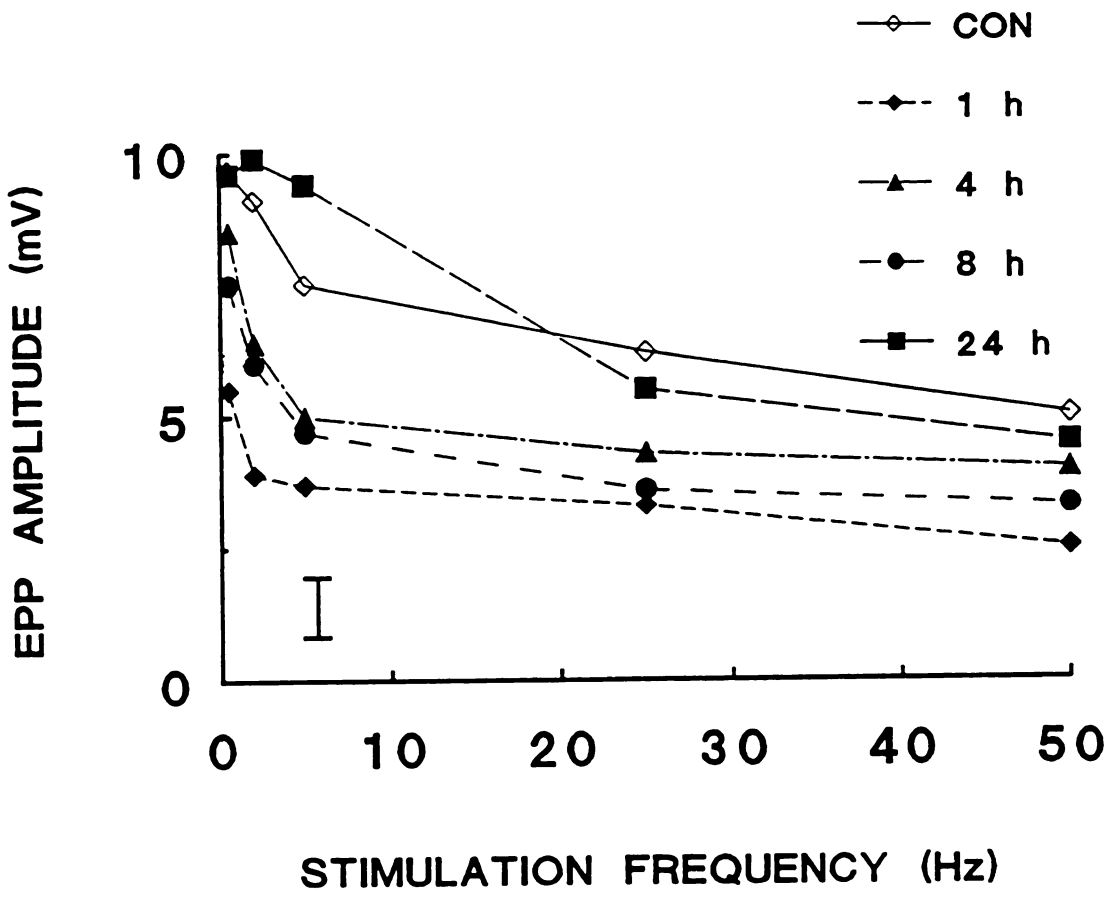


Figure 10. Effect of acute DTB treatment on EPP amplitude.

Effects of acute DTB treatment on EPPs at the rat neuromuscular junction. Diaphragms were removed 1, 4, 8, and 24 hour after treatment with DTB (25 mg/kg of DTB, ip) or vehicle. EPPs were recorded at stimulation frequencies of 0.5, 2, 5, 25, and 50 Hz from preparations in which the muscle was cut to prevent contraction due to stimulation of the phrenic nerve. Values are the means of at least four different preparations. The bar in the lower left corner is the average SEM. SEM values ranged from 0.3 to 3.3.

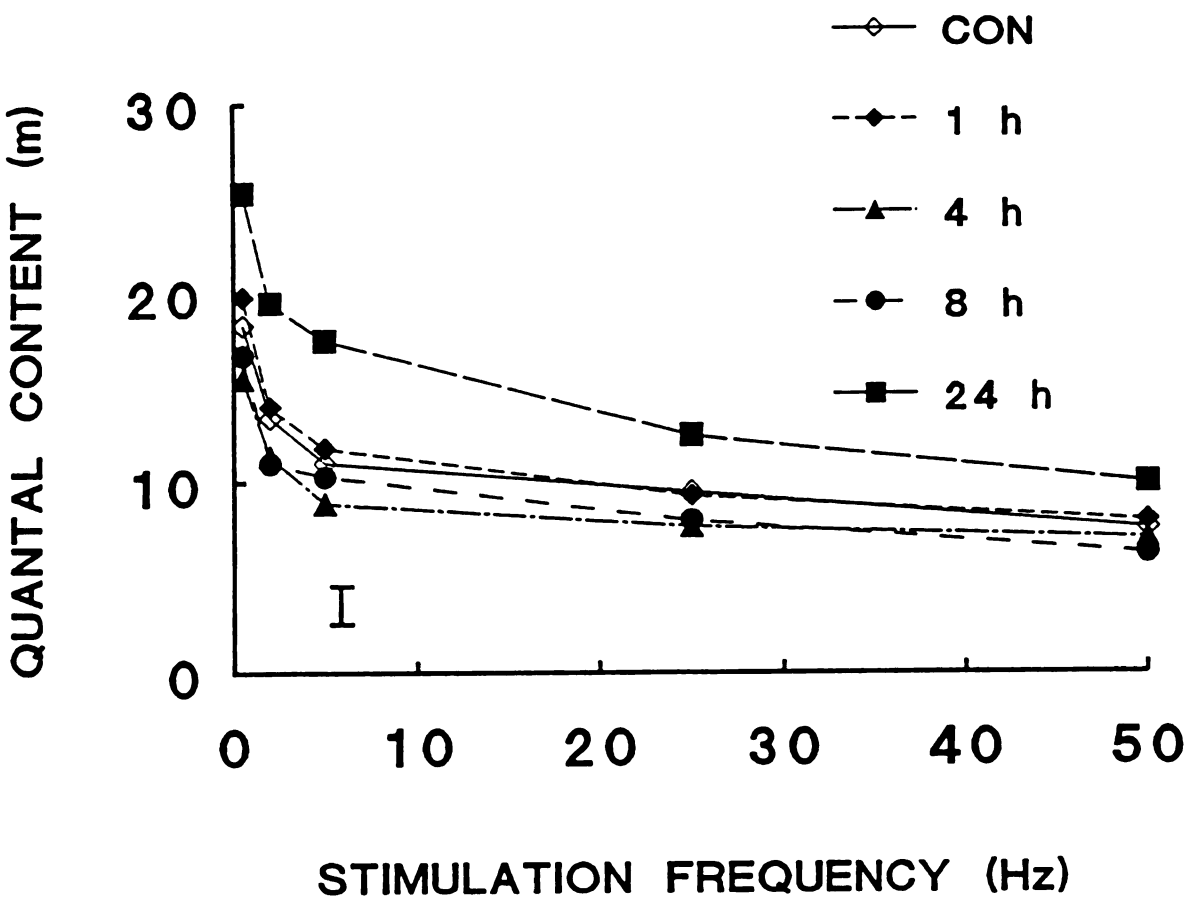


Figure 11. Effect of DTB treatment on *m* of evoked EPPs.

Effects of acute DTB treatment on quantal content at the rat neuromuscular junction. EPPs were recorded at stimulation frequencies of 0.5, 2, 5, 25, and 50 Hz from preparations in which the muscle was cut to prevent contraction due to stimulation of the phrenic nerve. Quantal content was calculated using the mean value method. The bar in the lower left represents the average SEM; SEM values ranged from 0.4 to 3.7.

EPP amplitude from muscles taken 1 hour after treatment with DTB appeared to be decreased compared to control, however, this was not significant. EPP amplitudes from muscles taken 4, 8 and 24 hour after treatment were similar to those of control muscles (Figure 10). Comparisons of quantal content demonstrated no difference between control and treated groups at any treatment time or any stimulus frequency (Figure 11).

MEPP frequency was significantly depressed in muscles taken 1 hour after treatment with DTB, while for muscles taken at 4, 8 and 24 hour after treatment these values had returned toward control levels (Figure 12). Even though MEPP amplitude was not significantly altered in muscles taken 1 hour following treatment with DTB there appeared to be a greater number of MEPPs with amplitudes of 1 mV or more (2.5% 1 hour after treatment compared to 1.4% in control muscles) and these values tended to occur at multiples of mean MEPP amplitude (Figure 13).

Rise and decay times for EPPs were unaffected by DTB treatment while rise and decay times for MEPPs were significantly prolonged at treatment times longer than 4 hour (Figure 14).

Effect of bath-applied DTB on EPPS and MEPPs. Due to the long equilibration time needed following removal of the diaphragm and transection of the muscle fibers, it was difficult to examine very early effects of DTB on transmission in diaphragms taken from treated rats. Therefore, diaphragms from untreated control rats were exposed to DTB in the bathing medium, thus allowing examination of very early potential effects of DTB on neuromuscular transmission.

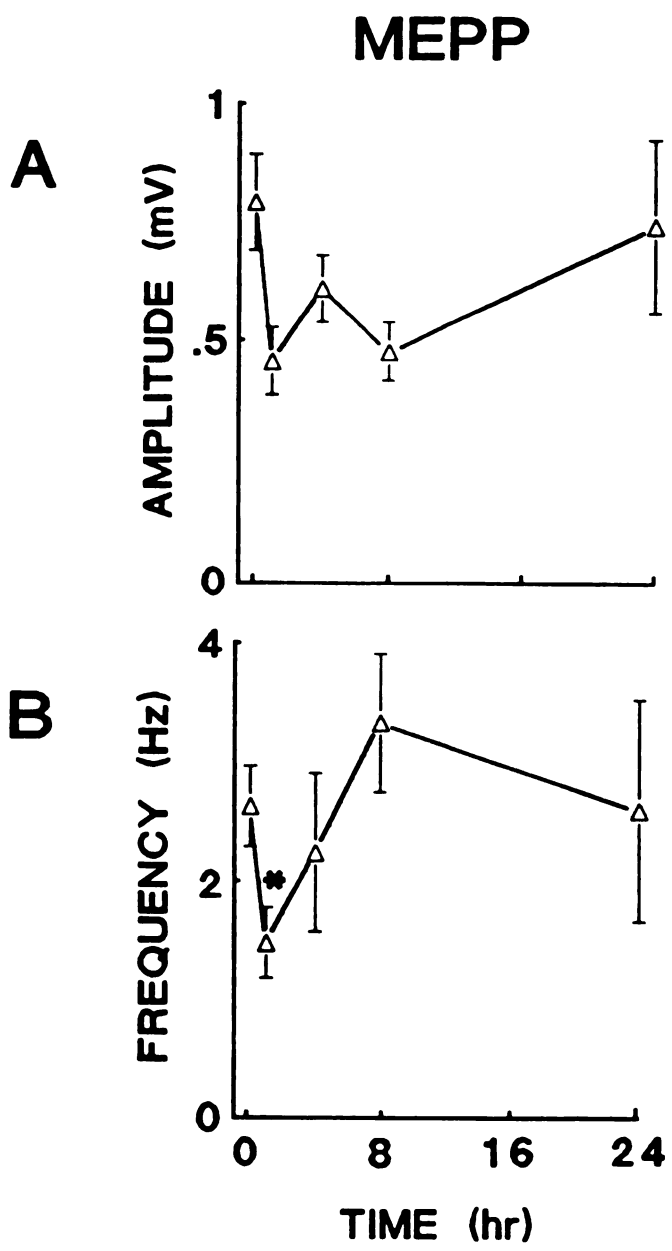


Figure 12. Effect of DTB treatment on MEPPs.

Effects of a single injection of DTB on amplitude (top) and frequency (bottom) of MEPPs at the rat neuromuscular junction. Rats were treated with 25 mg/kg of DTB, ip, and diaphragms were removed 1, 4, 8, and 24 hour after treatment with DTB or vehicle. MEPPs were recorded for 5 minutes prior to stimulation of the motor nerve for EPP recording. Values are the means ± SEM of at least four preparations. The asterisk indicates a significant decrease in MEPP frequency from control values ($p \leq 0.05$).

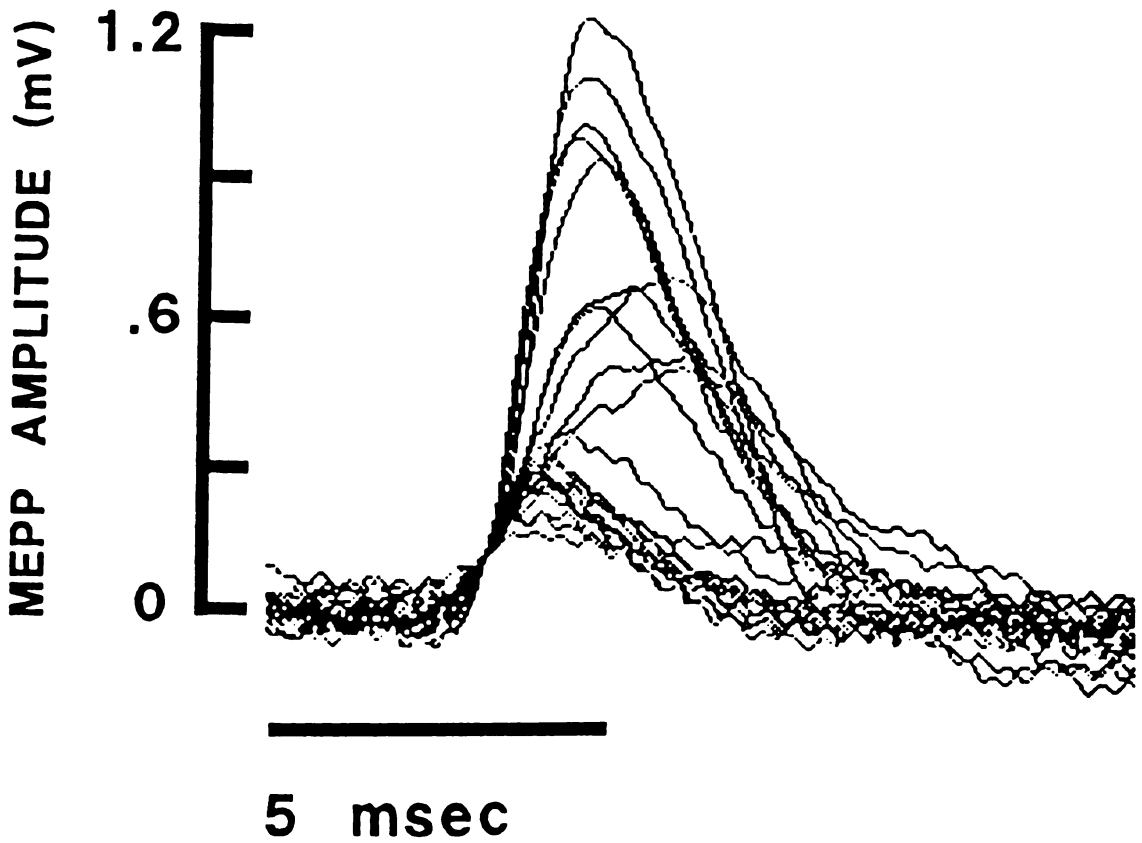


Figure 13. DTB treatment increases the incidence of abnormal MEPPs.

MEPPs recorded from a hemidiaphragm removed 1 hour following a single dose of 25 mg/kg of DTB. MEPPs were recorded for 5 minutes, prior to recording EPPs at various frequencies. The figure contains 25 MEPPs which were recorded consecutively and superimposed for the purpose of display. MEPP amplitude was 0.35 ± 0.02 mV (mean ± SEM) for this experiment with the majority of MEPPs being approximately 0.3 mV in amplitude (nonstandardized value); however, MEPPs with amplitudes of approximately 0.6 and 0.9 mV were also observed.

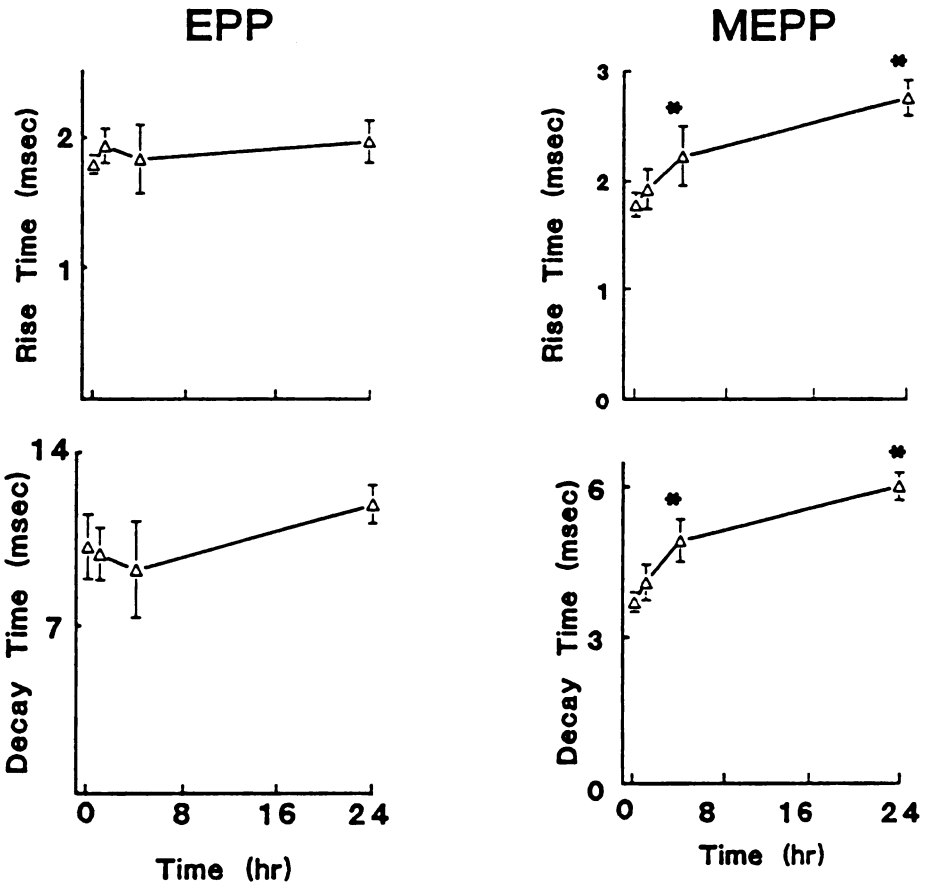


Figure 14. Effect of DTB treatment on rise and decay times for EPPs and MEPPs.

Effects of a single injection of DTB on rise and decay times for EPPs (left panel) and MEPPs (right panel) recorded at the rat neuromuscular junction. DTB treatment and uncoupling of muscle contraction from nerve stimulation are as in Figure 10. Rise and decay times were calculated from EPPs elicited at a frequency of 0.5 Hz. Values are the means ± SEM of at least four preparations. The asterisk indicates a value significantly different than control ($p \leq 0.05$).

The effects observed following exposure to 1.85 mM DTB were similar to those observed following exposure to 200 μM DTB except that they occurred at earlier times during exposure. Figure 15 shows that exposure of diaphragms to 1.85 mM DTB had a biphasic effect on EPP amplitude. During the first minutes of exposure there was an increase in EPP amplitude, followed by a decrease with continued exposure. Block of the EPP was observed in 4 out of 7 preparations after approximately 10 minutes of exposure to 1.85 mM DTB, while the mean time to block following exposure to 200 μM DTB was 30 min. The block of the EPP following exposure to DTB was readily reversed by changing the perfusing solution to a DTB-free buffer and could be reproduced by washing in a DTB-containing buffer a second time. In experiments in which block of the EPP was observed, there was a tendency for EPPs to return following rest periods during which stimulation was terminated to record MEPPs. In several experiments in which block of the EPP was reversed by washing with DTB-free buffer, the amplitude of the EPPs decreased with continued stimulation. If stimulation was stopped, the amplitude of EPPs evoked following a short rest period was increased. During block of the EPP there was a progressive decline in EPP amplitude, yet normal MEPPs were still observed. Therefore it appeared as though there was a steady decline in quantal content leading up to the observed block.

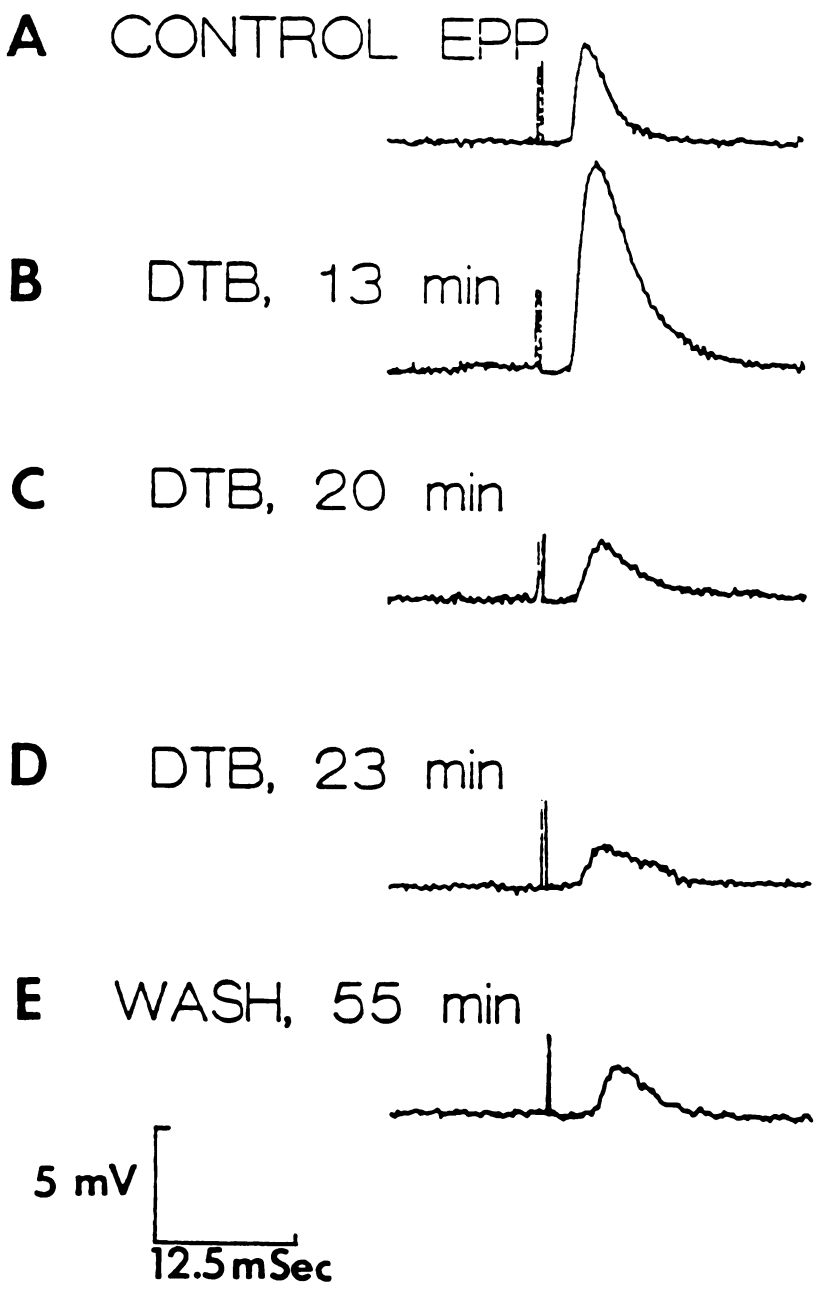


Figure 15. Effect of bath exposure to DTB on EPP amplitude.

Effects of bath application of DTB on nerve-evoked EPP amplitude. (A) Control EPPs were recorded for 4 minutes prior to bath application of DTB (1.85 mM). Following application of DTB there was (B) an initial rise in EPP amplitude followed by (C) a decrease in amplitude. (D) With continued exposure to DTB, EPP rise and decay times are increased. (E) Washing with DTB-free solution caused a partial reversal of DTBs effects.

MEPP frequency also exhibited a biphasic response following exposure to DTB. Soon after exposure to 1.85 mM DTB there was a large increase in MEPP frequency. With continued exposure, however, MEPP frequency declined toward control levels (Figure 16). At the time of block of the EPP, MEPP frequency was still significantly elevated (Figure 17).

As was observed in muscles from rats treated with a single large dose of DTB, large amplitude MEPPs were observed which occurred at multiples of the mean MEPP amplitude (Figure 18).

Diaphragms exposed to DTB in the perfusion medium also showed a slowing of the decay time for MEPPs (Figure 19).

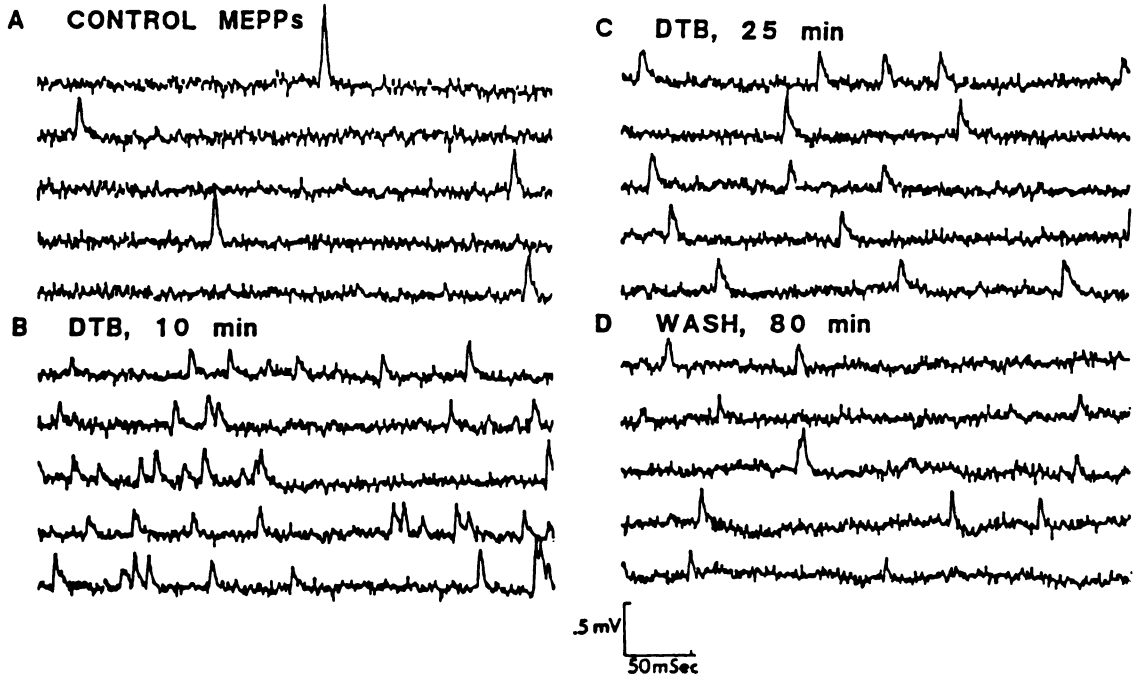


Figure 16. Effect of bath exposure to DTB on MEPP frequency.

Figure 16. Increased MEPP frequency produced by bath application of DTB (1.85 mM) at the rat neuromuscular junction. MEPPs were recorded (A) 5 minutes prior to bath application of DTB (control); (B) after 10 minutes exposure to DTB; (C) immediately following block of the EPP which occurred between 23 and 24 minutes after washing in DTB; or (D) after 80 minutes of washing with DTB free solution.

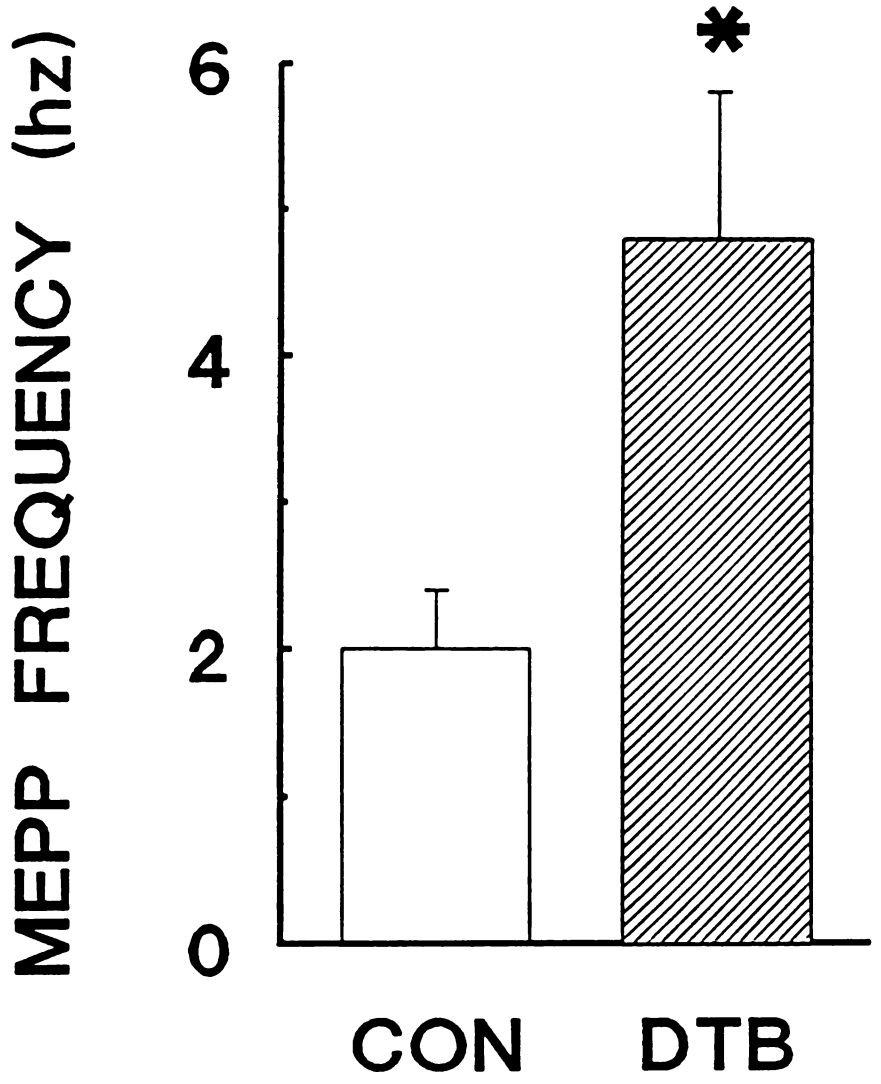


Figure 17. Bath exposure to DTB increased MEPP frequency.

Effect of bath-applied DTB (1.85 mM) on MEPP frequency at the rat neuromuscular junction. DTB treatment and uncoupling of contraction from nerve stimulation are as in Figure 13. MEPPs were recorded for 5 minutes prior to DTB application (control) and then at 10 minute intervals during bath application of DTB. Data presented are taken at a mean time of 35 minutes of exposure to DTB. Values are the mean ± SEM of seven preparations. The asterisk indicates a value significantly different than control ($p \leq 0.05$).

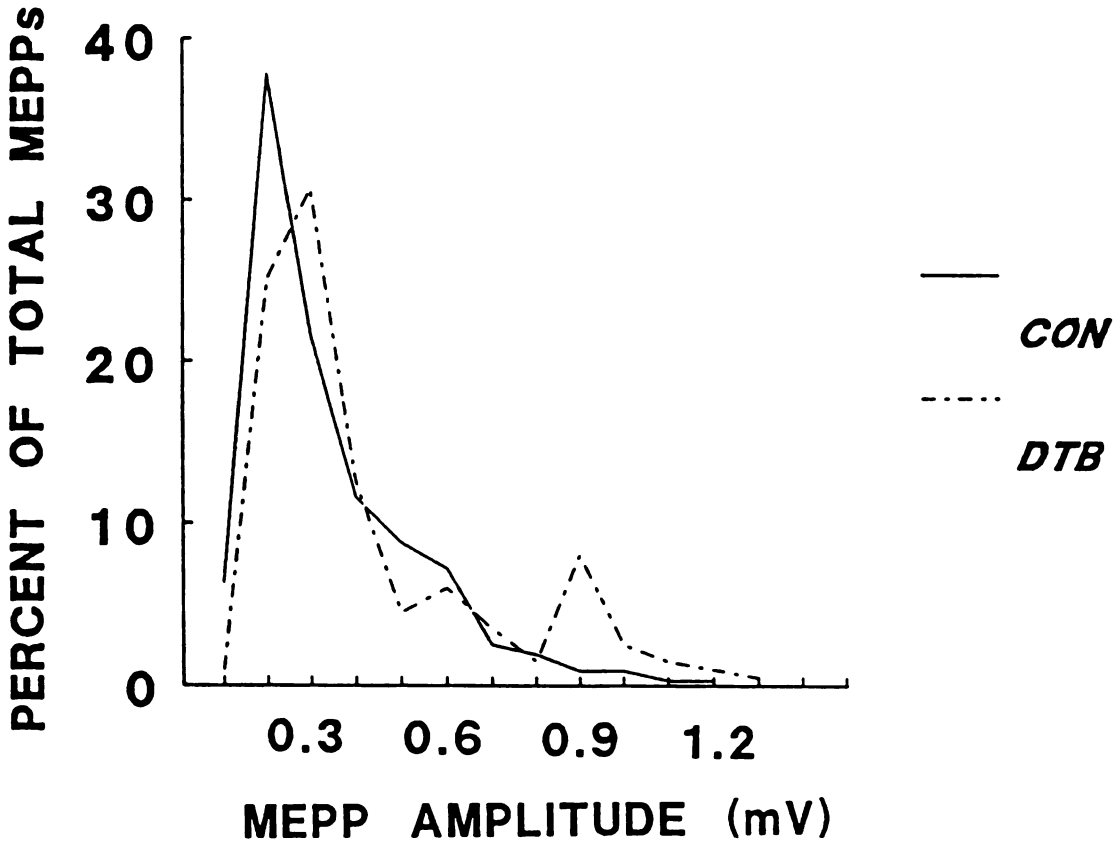


Figure 18. Bath exposure to DTB alters the distribution of MEPP amplitudes.

MEPP amplitude histograms before and after bath application of 1.85 mM DTB. Control MEPPs were recorded for 5 minutes at the start of each experiment. MEPPs recorded in DTB-containing medium were measured at a mean time of 33.5 minutes following exposure to DTB. The data are displayed as percent of total MEPPs observed at each amplitude. MEPP amplitudes are not standardized to -50 mV.

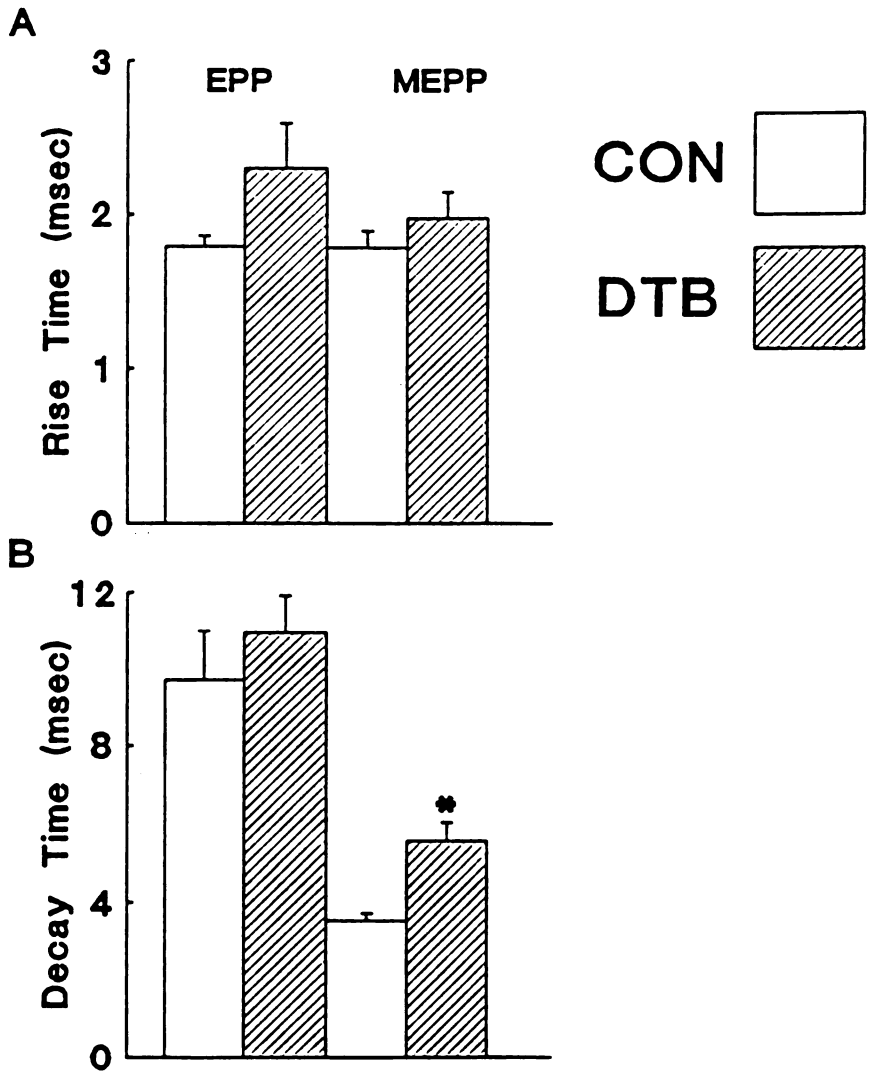


Figure 19. Effect of bath exposure to DTB on rise and decay times for EPPs and MEPPs.

Effects of bath-applied DTB on (A) rise and (B) decay times of MEPPs and EPPs at the neuromuscular junction. Diaphragms were removed from untreated animals, cut, and placed in [low K^+] buffer to prevent contraction due to stimulation of the phrenic nerve. MEPPs and EPPs were recorded before and after bath application of DTB (1.85 mM). EPPs were evoked at a frequency of 1 Hz. Diaphragms were maintained in DTB-containing solution until block of the EPP occurred or for approximately 60 min, whichever occurred first. Data presented are taken at a mean time of 33.5 minutes of exposure to 1.85 mM DTB. Values are the means ± SEM of at least six preparations. The asterisk indicates a value significantly different than control ($p \leq 0.05$).

Discussion.

Chronic daily treatment with DTB causes flaccid muscle weakness and depressions in evoked and spontaneous release of ACh. Conversely, acute administration of DTB does not cause flaccid neuromuscular weakness, even when given at doses which approach the LD_{50}. What was unknown was whether acute administration of DTB could alter neuromuscular transmission in subtle ways that might not be apparent in the whole animal over that time course. In striated muscle, a twitch will occur so long as the amplitude of the EPP reaches threshold for generation of an action potential. Typically, a large safety-factor exists for neuromuscular transmission (Paton and Waud, 1967). That is, the amplitude of the evoked EPP is typically much greater than that necessary to elicit an action potential in the muscle (and hence a twitch). A reduction in safety factor for neuromuscular transmission during acute DTB-treatment could reduce the EPP amplitude compared to non-treated end-plates yet not reduce the amplitude below the action potential threshold, and hence not block muscle contraction. This is particularly true for the diaphragm, which has a much higher safety factor than do hindlimb muscles (Paton and Waud, 1967; Waud and Waud, 1972). This no doubt, accounts for the observation that paralytic effects of DTB in the hindlimb appear to precede those on respiratory muscles (Atchison and Peterson, 1981). By examining early effects on neuromuscular transmission, in rats treated with a single 25 mg/kg dose of DTB, or very early effects on transmission following bath application of DTB, it was hoped that events responsible for the onset of paresis in chronically treated rats could be

determined. In general the effects of bath application of DTB on neuromuscular transmission resembled those following a single large dose of DTB, the main difference being that following bath application there was an initial transient increase in EPP amplitude, MEPP frequency and MEPP amplitude followed by a steady decline with continued application of DTB, whereas following a single large dose of DTB only a decrease was noted.

With the exception of decrease of quantal content, the alterations in transmission observed following a single large dose were similar to those observed following chronic treatment with DTB. These effects included depression of EPP amplitude (not significant), a decrease in MEPP frequency (Weiler et al., 1986; Atchison, 1989) and a slowing of the rise and decay times for MEPPs (Atchison, 1989). Atchison (1989) found the decrease of quantal content to be due to a decrease in the readily releasable store of ACh rather than effects on the probability of release. The fact that neither a reduction in quantal content nor paresis was observed in acutely treated rats may indicate that the paresis observed in chronically treated rats is due to a gradual decrease in the releasable pool of transmitter.

In both the bath application and acute dose studies a slowing of rise and decay times for EPPs and MEPPs was observed which did not recover to control levels at the same rate as MEPP frequency and EPP amplitude. Atchison (1989) also reported a similar alteration of the rise and decay times following chronic exposure to DTB. These alterations of rise and decay times may indicate that DTB has postsynaptic effects in addition to the presynaptic effects reported by others

(Atchison et al., 1982; Weiler et al., 1986). A postsynaptic effect would be expected if DTB were reacting with sulfhydryl groups located on the AChR (Karlin and Bartels, 1966; Karlin, 1980). Reduction (Ben-Haim et al., 1973; 1975; Terrar, 1978), oxidation or sulfonation (Steinacker and Zuazaga, 1981; 1987) of the postjunctional nicotinic receptor all cause alterations of decay kinetics of the EPP or EPC.

In conclusion, acute exposure to DTB via a single large dose or bath application causes deficits in neuromuscular transmission. With the exception of a decrease in quantal content the effects observed following acute exposure in many ways resemble those observed in rats paralyzed following chronic treatment with DTB. From these results it appears that alterations in neuromuscular transmission occur soon after treatment with DTB is initiated and that with continued exposure these deficits progress to the more generalized muscle weakness observed in chronically treated rats.

Chapter 6. Effects of exposure to DTB on end-plate currents.

Summary of results of acute studies. In experiments designed to determine early effects of DTB on junctional transmission, it was found that as early as 1 hour following a single dose 25 mg/kg of DTB, at a time when no muscle weakness is observed, EPP amplitude appears depressed (not significant) and MEPP frequency was decreased (Chapter 5). Rise and decay times for MEPPs were prolonged in a manner similar to that observed in chronically-treated rats exhibiting skeletal muscle weakness induced by DTB. The early effects of DTB on EPP amplitude and MEPP frequency appear to reverse by 24 hour after a single dose of DTB; the slowing of rise and decay times does not. These early alterations of rise and decay times may represent effects of DTB on transmission which, with continued exposure, could lead to the decreased junctional transmission observed in chronically-treated rats.

Prolongation of rise and decay times of synaptic potentials could be due to presynaptic or postsynaptic effects (Table 1). Prolonged rise and decay times for EPPs and MEPPs might be observed if DTB altered binding of ACh to its receptor, opening and closing of the receptor-gated ionic channel or reduced levels or activity of AChE (Eccles and MacFarlane, 1949). A DTB-induced depolarization of the muscle cell membrane would cause a decrease in EPP and MEPP amplitude as well as alter time constant for decay of current flowing through the ACh-channel (Magleby and Stevens, 1972).

Table 1. Summary of effects of DTB.

	Acute treatment		Chronic treatment	
	Presynaptic		Postsynaptic	
Muscle weakness	no	yes	?	?
EPP (*m*)	-	dec.	yes	-
Failure of transmission	no	yes	yes	-
MEPP frequency	dec.	dec.	yes	?
Slow rise and decay for potentials	yes	yes	?	?
Abnormal MEPPs	yes	yes	?	?

(Weiler et al. (1986) found no evidence of alterations of either postjunctional membrane potential or muscle input resistance in rats paralyzed after 5 days of DTB treatment.) A decrease in MEPP amplitude could lead to an apparent decrease in MEPP frequency by increasing the number of MEPPs lost in background noise. DTB could inhibit the packaging of ACh into vesicles thus decreasing quantal size (MEPP amplitude) and in turn EPP amplitude (without affecting *m*). DTB-induced terminal retraction or glial cell interposition [pathological observations associated with chronic treatment with this agent (Kemplay, 1984; Jones, 1989)] could also prolong rise and decay times by altering the diffusion distance for ACh within the synaptic cleft to the end-plate receptors. DTB might also cause release of ACh from sites in the terminal which are not directly apposed to postjunctional invaginations again increasing the diffusion pathway for ACh to its receptors.

Objectives. The objectives of the studies described in the following chapters were to determine whether a postsynaptic modification was responsible for the observed effects on EPP and MEPP rise and decay kinetics. More specifically, the purpose of these studies was to determine whether DTB altered current flow through AChR-gated channels in muscles taken from rats exposed acutely to a dose of DTB which causes no overt signs of neuromuscular weakness at the whole animal level or chronically to DTB at a regimen that causes observable neuromuscular weakness.

Hypothesis

Treatment of rats with DTB leads to an alteration in current flow through AChR-gated ion channels present in the muscle cell membrane. The time course of these effects indicate that they may be partially responsible for the weakness observed with continued treatment.

Introduction to voltage clamp. Following a change in voltage across a membrane or a change in conductance of channels in a membrane there will be a current (I_t) flowing across the membrane. The measured current has two components- a capacitive component (I_c) and an ionic component (I_i), where:

$$I_t = I_c + I_i.$$

In this equation the ionic component (I_i) is equal to the capacitance of the membrane times the change in voltage across the membrane as a function of time (dv/dt). By clamping the membrane potential at a constant level the change in voltage will equal zero, so the capacitive current component drops out of the above equation. This allows measurement of only the ionic current flowing through channels in the membrane.

The voltage clamp which was used in these studies utilizes two microelectrodes placed in the same cell to maintain the membrane potential at a steady level. One electrode is used to measure membrane potential via a voltage follower circuit and the second electrode passes current back into the cell. The voltage clamp amplifier compares the membrane potential measured by the voltage follower circuit to a reference voltage (holding potential). Current is passed through the second electrode to bring the membrane potential back towards the reference or clamped level (Halliwell et al., 1987). Following transmitter release into the synaptic cleft, during which time an EPP or MEPP would normally be recorded, the

voltage follower circuit senses a change in membrane potential which leads to the passage of current through the current passing electrode. The current required to clamp the membrane potential at a constant level during the action of transmitter can then be recorded. This current should be equal and opposite to that flowing through AChR-channels in the membrane during the action of transmitter.

Utilizing voltage clamp it is possible to determine several characteristics of ionic currents flowing through the channels within the membrane. First, the peak current moving through channels at a given membrane potential can be measured. By measuring the peak current which flows across the membrane at a series of holding potentials a current vs voltage relationship can be obtained (Takeuchi and Takeuchi, 1959). Several pieces of information can be gathered from the current vs voltage relationship. The slope of current vs voltage relationship can give information concerning the conductance of channels active during the measured current. Second, the membrane potential at which the end plate current (EPC) reverses its direction of flow through the membrane (reversal potential) can be determined. This provides information on the nature of the ions which make up the active current (Takeuchi and Takeuchi, 1960). In addition, by examining the rise and decay phases of single EPC's or MEPC's one can gain information regarding the kinetics of channel opening and closing (Magleby and Sevens, 1972a; 1972b; Linder et al., 1984).

Methods.

The methods used for the following experiments are described in detail in the materials and methods section of this manuscript. Briefly, voltage clamp experiments were conducted using the two microelectrode voltage clamp technique (Takeuchi and Takeuchi, 1959) and hemidiaphragm preparations (Bülbring, 1946) taken from control male rats (180-250 g, Harlan Sprague-Dawley Laboratories, Madison, WI) and rats exhibiting muscle weakness following chronic treatment with DTB (1 mg/kg/day) or rats exhibiting no weakness following a single dose of DTB (25 mg/kg).

Results

Effects of DTB on behavior of rats. Rats treated with 1 mg/kg/day of DTB for 7 to 8 days exhibited marked muscle weakness and displayed other signs of DTB intoxication (Atchison and Peterson, 1981; Atchison et al., 1981a; Williams et al., 1986). Rats given a single 25 mg/kg dose of DTB failed to develop any observable weakness for up to 24 hour after treatment and have been shown not to develop weakness for extended periods of time following treatment (Atchison and Peterson, 1981), however, these rats did develop other signs of DTB intoxication observed in chronically treated rats.

Effects of DTB on EPC amplitude and quantal content and MEPC amplitude. Figure 20 shows peak amplitude of EPCs recorded at holding potentials between -100 mV and +10 mV. In control cut muscles, the EPC reversal potential occurred at

a holding potential of approximately -10 mV, a value similar to that reported by Glavinovic (1979) for cut muscle of the rat, while the current vs voltage relationship had a slope of 2.26 nA/mV. In several control preparations there was a inward rectification of the current vs voltage relationship at holding potentials more negative than -60 mV, a result consistent with previous observations of voltage-dependence of the EPC at hyperpolarizing potentials for frog neuromuscular junctions (Magleby and Stevens, 1972b). The current vs voltage relationship for muscles taken from rats following either acute high dose or chronic low dose treatment with DTB exhibited a similar pattern to that of controls. The slope of the current vs voltage relationship was decreased to 1.48 and 1.71 nA/mV in muscles taken 1 and 24 hour respectively following treatment with a single dose of 25 mg/kg and to 1.1 nA/mV in muscles from chronically-treated rats. In DTB-treated preparations in which inward rectification of the current vs voltage relationship was observed, the holding potential at which rectification occurred was more negative than in control preparations, typically occurring in the range of -65 to -75 mV.

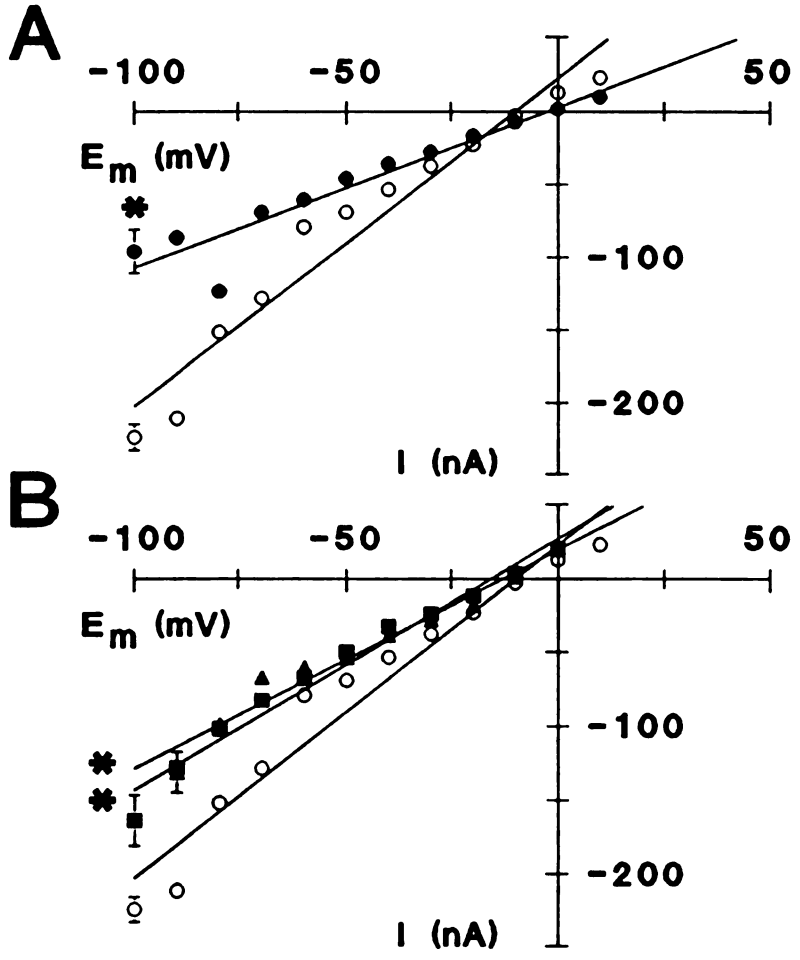


Figure 20. Effect of DTB treatment on peak EPC amplitude.

Effects of DTB on peak end-plate current (EPC) amplitude at membrane potentials held between -100 mV and +10 mV. EPC amplitude recorded in hemidiaphragm preparations taken from A) (o) control rats and (●) rats exhibiting neuromuscular weakness following chronic treatment for 7 days with 1 mg/kg/day, ip. of DTB or B) (o) control rats and rats treated (▲) 1 hour and (■) 24 hour previously with a 25 mg/kg dose of DTB. Values are the mean of 3-11 preparations. The error bar represents the average SEM for all values of a given group. The asterisk (*) denotes a slope significantly different from control ($p < .05$).

Quantal content (m) of EPCs was calculated for EPCs elicited at holding potentials between -60 and -30 mV. Values of m calculated at holding potentials more negative than -60 mV were not used due to rectification of the EPC observed in some preparations, while values for m calculated at holding potentials more positive than -30 mV were not used. This was due to the fact that normal amplitude MEPCs were lost in background noise in some preparations. Giant MEPCs, which were more common in muscles from DTB-treated rats, would still be counted thus biasing mean MEPC amplitude towards the giant MEPC amplitudes. Figure 21 shows that in muscles taken from rats exhibiting paresis following DTB treatment there was a significant decrease ($p < 0.05$) in m. Although m also appears depressed in muscles removed 1 and 24 hour following a single 25 mg/kg dose of DTB, this decrease is not significant ($p > .05$).

In control preparations, mean amplitude of MEPCs recorded at a holding potential of -50 mV was 1.93 ± 0.31 nA, a value similar to that reported for transected rat diaphragm muscle by Glavinovic (1979). In muscles taken from rats 1 and 24 hour following treatment with a single 25 mg/kg dose of DTB, mean MEPC amplitude at -50 mV was 2.75 ± 0.7 and 2.02 ± 0.73 nA respectively, while in muscles taken from rats chronically-treated with DTB, MEPC amplitude was 1.96 ± 0.3 nA at -50 mV. None of these values differed significantly from control. As early as 1 hour following treatment with a 25 mg/kg dose of DTB, the incidence of abnormally large (greater than twice the mode value), slow MEPCs was increased from 5.1% of all MEPCs in controls to 28.4% in muscles taken 1 hour after

treatment (Figure 22). Moreover the incidence of these abnormally large MEPCs was increased to 32.4% of all MEPCs in muscles taken from rats treated 24 hour previously with a 25 mg/kg dose of DTB. As described earlier in current clamp experiments (Atchison, 1989), the frequency of large slow MEPCs was also increased in muscles taken from rats treated chronically with DTB (12% were greater than twice the mode amplitude for controls). In addition to the observed increase in abnormally large, slow MEPCs, there also appears to be an increase in the number of small MEPCs with amplitudes of 0.25 to 0.75 nA (at -50 mV) both in muscles from rats treated 24 hour previously with a 25 mg/kg dose of DTB and in muscles from rats chronically treated with DTB (Figure 22). To determine whether DTB treatment decreased the amplitude of normal MEPCs, as was reported for MEPPs earlier (Weiler et al., 1986; Spitsbergen and Atchison, 1990), we examined the amplitudes of MEPCs with fast rise and decay which appeared to be the mode of the amplitude distribution, and hence presumably reflected single quantum MEPCs. In both control and treated preparations the amplitude of the mode MEPCs was slightly smaller than the mean MEPC amplitudes, however, the amplitude of mode MEPCs in muscles taken from rats 1 hour following a 25 mg/kg dose of DTB was greater than that for mode MEPCs in controls ($p < 0.05$; Figure 23).

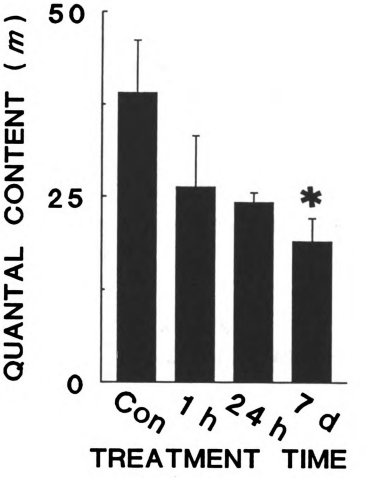


Figure 21. Effect of DTB treatment on *m* of EPCs.

Effects of DTB on quantal content (*m*) of EPCs recorded in hemidiaphragm preparations taken from control rats, rats 1 and 24 hour following a single 25 mg/kg dose of DTB, or from rats treated for 7 days with 1 mg/kg/day, ip. of DTB. Quantal content was calculated by dividing mean EPC amplitude by mean MEPC amplitude for EPCs and MEPCs recorded from the same end-plate region at the same holding potential. Values are the means ± SEM of 3 to 11 preparations. The asterisk (*) denotes values significantly different from control ($p < .05$).

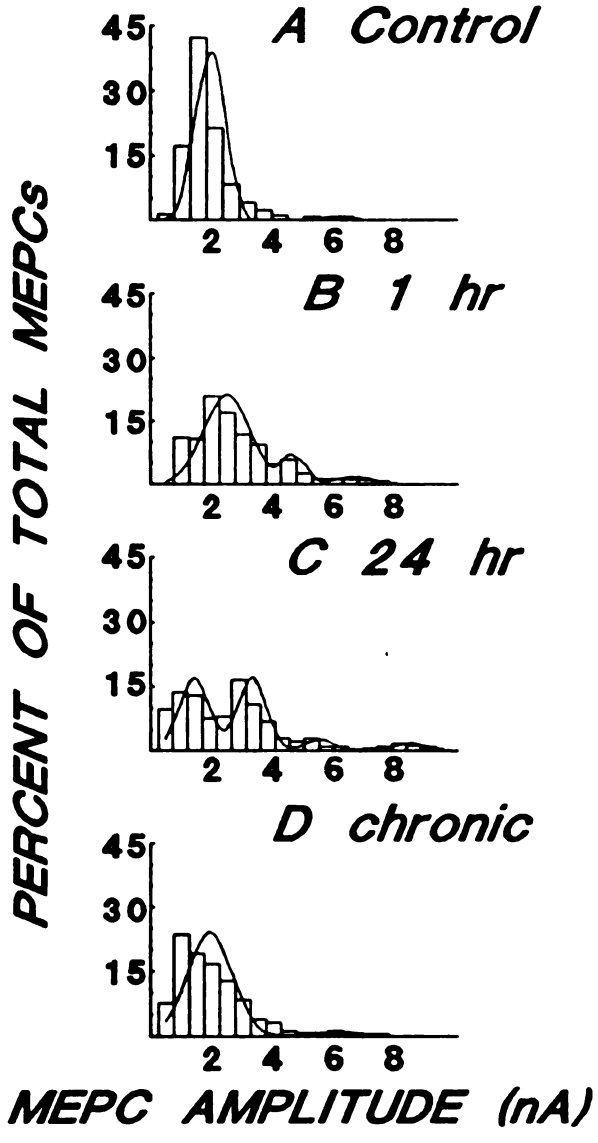


Figure 22. Effect of DTB treatment on the distribution of MEPC amplitude.

Amplitude distribution histogram of MEPCs recorded at a holding potential of -50 mV in hemidiaphragms taken from A) control rats, and rats treated 1 B) and 24 C) hour previously with a 25 mg/kg dose of DTB, respectively and D) rats treated chronically with 1 mg/kg/day of DTB for 7 days. Values are expressed as percentage of total number of MEPCs counted. Between 100-200 MEPCs were sampled from at least 3 preparations for each treatment group.

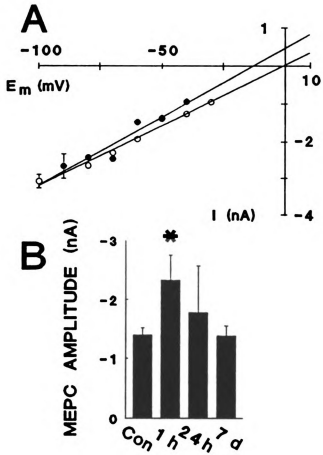


Figure 23. Effect of DTB treatment on the amplitude of modal MEPCs.

Effects of DTB on mean MEPC amplitude for modal (single quantum) MEPCs. MEPCs having the modal value from each end-plate region were summed and the effects of DTB following acute and chronic exposure were determined. A) Current/Voltage relationship for MEPCs recorded in hemidiaphragms taken from (o) control and (•) chronically-treated rats measured at holding potentials from -20 to -90 mV. B) Comparison of MEPC amplitudes recorded at a holding potential of -50 mV in muscles taken from control rats, rats treated 1 and 24 hour previously with a single 25 mg/kg dose of DTB or rats exhibiting neuromuscular weakness following 7 days of treatment with 1 mg/kg/day of DTB. Values in A are the means of at least 9 preparations, the single error bar represents the average SEM for each group. Values in B represent the means ± SEM of 3 to 11 preparations. Between 50 and 100 MEPCs were sampled at each holding potential in a given preparation. The asterisk (*) indicates a value significantly different from control ($p < 0.05$).

Effect of DTB on rise and decay of synaptic potentials. Despite the reported slowing of decay time for EPPs described earlier, the decay time constant (τ) for EPCs was not greater in muscles from treated rats than controls. In fact, τ_{EPC} for muscles taken 24 hour following a single 25 mg/kg dose of DTB was *decreased* compared to controls (Figure 24). Initial examination of τ_{MEPC} indicated a significant increase ($p < 0.05$) in τ for muscles taken from rats exhibiting neuromuscular weakness following chronic treatment with DTB and a reversal of the normal voltage-dependence of τ (Figure 25). τ_{MEPC} for muscles taken following acute treatment with DTB was unchanged or only slightly increased. If one calculates τ for the mode MEPCs, leaving out the abnormally slow, large amplitude MEPCs, then τ_{MEPC} is actually significantly decreased ($p < 0.05$) in muscles from chronically-treated rats compared to controls (Figure 26) and the voltage-dependence of τ is normal. In a similar manner, τ_{MEPC} for mode MEPCs recorded at -50 mV in muscles taken 24 hour following a single 25 mg/kg dose of DTB is also decreased significantly ($p < 0.05$) analogous to the results seen for τ_{EPC} for these muscles.

The 10-90% rise time for EPCs in muscles removed 24 hour following a single 25 mg/kg dose of DTB is shorter than in controls (Figure 27), while 10-90% rise time for EPCs of muscles taken 1 hour following a single 25 mg/kg dose of DTB, or those taken from chronically-treated rats have rise times similar to controls. DTB treatment had no effect on MEPC rise time calculated for the modal MEPCs.

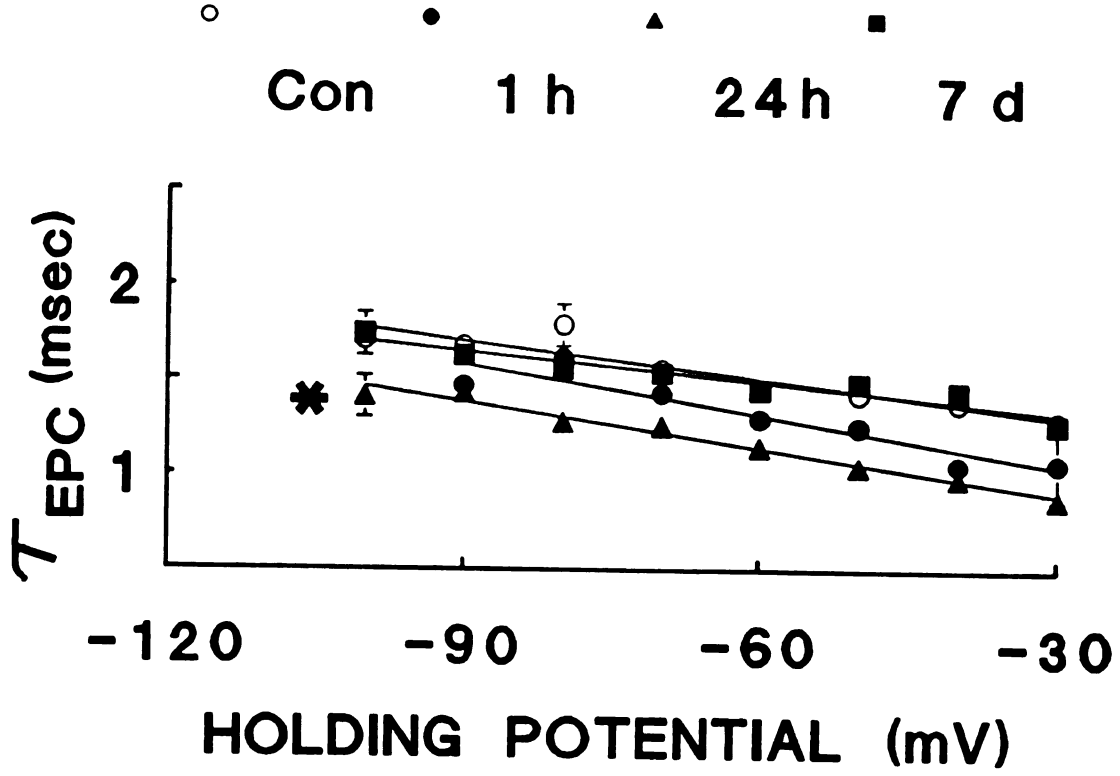


Figure 24. Effect of DTB treatment on τ_{EPC}.

Effects of DTB on τ_{EPC} and its voltage-dependence. Decay time constants (τ) were determined by a computerized curve fitting routine, which fit exponential curves to the decay phase of the EPCs. τ was determined for EPCs elicited at holding potentials from -30 to -100 mV in muscles from (o) control rats, in muscles taken (●) 1 and (▲) 24 hour following a single 25 mg/kg dose of DTB and muscles from (■) rats treated for 7 days with 1 mg/kg of DTB. Values are the means of 3 to 11 preparations. Error bars represent the average SEM for each group. In panel A the asterisk (*) denotes a line whose points have significantly different heights from those on the control line ($p < 0.05$), while in panel B the asterisk (*) denotes a value significantly different from control ($p < 0.05$).

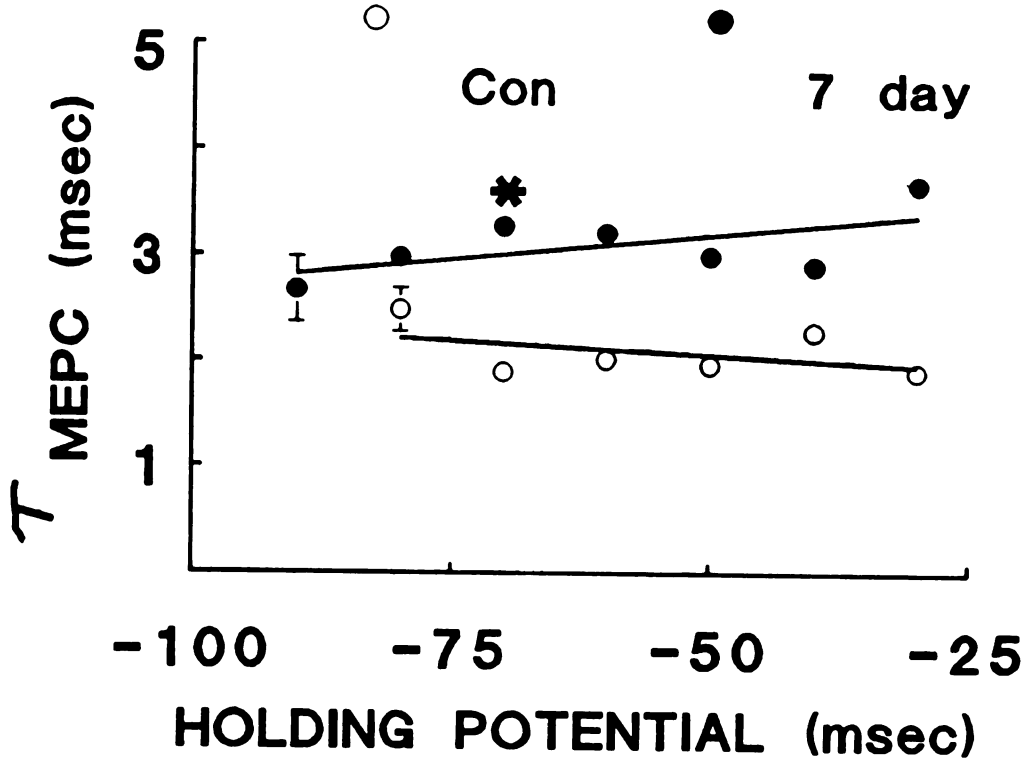


Figure 25. Effect of DTB treatment on τ_{MEPC}.

Effects of DTB on τ_{MEPC} and its voltage-dependence. Calculation of τ_{MEPC} was similar to that described for τ_{EPC} in Figure 24. MEPCs were recorded and τ calculated at holding potentials of -30 to -100 mV in muscles taken from (o) control rats and from rats (●) chronically treated with DTB. Values represent the mean ± the average SEM of at least 6 preparations. Values to the right of the asterisk (*) have heights significantly different from points on the control line ($p < 0.05$).

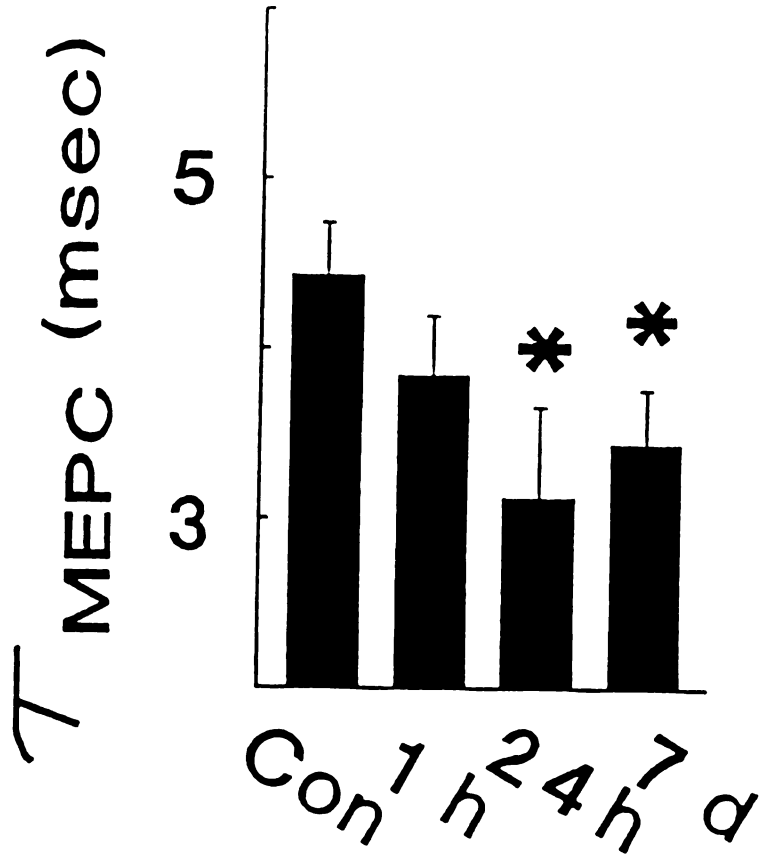


Figure 26. Effect of DTB treatment on τ_{MEPC} for modal MEPCs.

Effect of DTB on τ_{MEPC} and voltage-dependence of τ for modal (single quantum) MEPCs recorded at a holding potential of -50 mV. τ was calculated from the inverse of the slope of a semilog plot of the decay phase vs time for summed MEPCs having the mode amplitude from a given end-plate region. Values represent the means ± SEM of at least 3 preparations. The asterisk (*) denotes a value significantly different from control ($p < 0.05$).

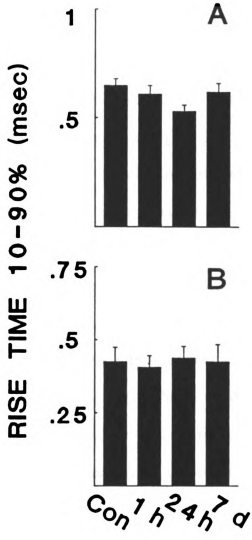


Figure 27. Effect of DTB treatment on 10-90% rise time for EPCs and modal MEPCs.

Effects of DTB on the 10-90% rise time of A) EPCs and B) mode amplitude MEPCs. The 10-90% rise time was calculated for EPCs and MEPCs recorded at a holding potential of -50 mV in muscles taken from control rats, chronically treated rats and acutely treated rats. Values represent the mean ± SEM of at least 3 preparations.

Discussion.

Although rise and decay times for EPPs and MEPPs were reported to be prolonged both in muscles from chronically-treated rats exhibiting DTB-induced muscle weakness (Atchison, 1989) and in muscles taken from rats treated acutely with a single 25 mg/kg dose of DTB (Spitsbergen and Atchison, 1990), a similar prolongation of rise and decay kinetics of EPCs and MEPCs was not observed in this study. Initially it appeared as though τ_{MEPC} was increased and the voltage-dependence of τ reversed in muscles from rats exposed to DTB. However, upon closer examination it became evident that this was an artifact caused by the increased incidence of abnormal, large amplitude MEPCs with slow rise and decay. This appears to be the case, since at depolarized holding potentials the slowing of decay kinetics was most pronounced, an observation which opposes the normal voltage-dependence of τ_{MEPC} observed in frog for the nicotinic ACh channel (Kordas, 1969; Magleby and Stevens, 1972a). If on the other hand, one calculates τ_{MEPC} only for MEPCs with fast rise and decay, which are still observed in every preparation, then MEPC decay does not appear to be slowed and the voltage-dependance of τ is normal. Therefore, what appears to be happening is that at depolarized holding potentials, at which MEPC amplitude is depressed due to a decreased ionic driving force across the membrane, a higher percentage of normal MEPCs is lost in background noise thus causing the ratio of abnormal large amplitude MEPCs to normal MEPCs to increase in the DTB-treated preparations. This apparent increase in the number of abnormal MEPCs then biases all further calculations of mean

MEPC amplitude and rise and decay kinetics toward the abnormal MEPCs.

It is interesting that once the abnormal MEPCs are removed from the calculation of τ_{MEPC} that τ actually appears to be decreased in muscles from rats exposed to DTB as does τ_{EPC} for muscles taken 24 hour following a single large dose of DTB. A DTB-induced decrease in decay kinetics would not be unexpected since DTB is a mild reducing agent (Preisler and Bateman, 1947) and reduction of the nicotinic AChR has been reported to cause shortening of decay kinetics for MEPPs and MEPCs (Ben-Haim et al., 1973; 1975; Terrar, 1978).

In light of the results described above it is apparent that the prolongation of rise and decay times observed for EPPs and MEPPs following exposure to DTB does not appear to be due to a DTB-induced prolongation of channel open-time. The observed prolongation of rise and decay times for MEPPs could easily be explained by the increase in the incidence of abnormal MEPPs observed following DTB exposure. In many cases the amplitudes of these MEPPs are no different from the normal MEPPs, with only the rise and decay times being prolonged, making it difficult to separate these events during analysis. In all cases, one is able to observe normal, rapid rise and decay MEPPs or MEPCs interspersed with the abnormal ones. Therefore, the cause of the observed slowing must not be due to an effect on all AChR-channel complexes. Instead DTB might alter a portion of ACh release sites or possibly a portion of the receptor sites, thus affecting only a fraction of the observed MEPCs. Although Weiler et al. (1986) reported no difference in the input resistance of the muscle membrane between DTB-treated rats and controls, an in-

depth study of passive cable properties was not made. It is possible that the reported slowing of MEPP and EPP rise and decay times could be due to a DTB-induced alteration in cable properties of the muscle membrane, an effect which would be lessened in a preparation for which the membrane potential is held constant and only active current flow measured.

The origin of these abnormally large amplitude, slow decay MEPCs remains an enigma. They are occasionally observed in control preparations, but both their incidence of occurrence and their characteristic amplitude and kinetics are much more pronounced in DTB-treated preparations. The events are observed under voltage-clamp interspersed with MEPCs having normal amplitude and decay kinetics. This makes it unlikely that the aberrant MEPCs are due to a generalized postjunctional phenomenon. It is certainly possible that a fraction of the ACh channels may be modified following treatment with DTB, leading to the occurrence of these abnormal quantal events. On the other hand, an equally likely, and perhaps more probable explanation is that these events reflect alterations by DTB in the quantal release process. Extremely large amplitude (>1.5 mV) MEPPs have been reported at mammalian neuromuscular junctions since the 1950's (Liley, 1956). Yet in some pathological states, the incidence of occurrence of the abnormal MEPPs is increased dramatically and their rise and decay time is prolonged markedly. These abnormal MEPCs, seen as giant, slow MEPPs in current clamp experiments have been reported to be present following treatment of neuromuscular junctions with botulinum toxin (Cull-Candy et al., 1976), 4-aminoquinoline (Molgo, and Thesleff,

1982), following prolonged curarization or paralysis with tetrodotoxin (Katz, 1966), or following nerve degeneration (Vizi and Vyskocil, 1979). The incidence of small amplitude MEPPs (sub-MEPPs) is also elevated at neuromuscular junctions poisoned with botulinum toxin (Cull-Candy et al., 1976) and at developing or regenerating neuromuscular junctions (Muniak et al., 1982). These abnormally large amplitude, slow MEPPs and small amplitude sub-MEPPs both appear to be associated with degeneration and regeneration of the terminal region. In DTB-treated rats the incidence of large, slow MEPCs is elevated as early as 1 hour following treatment, while the incidence of both large and small amplitude MEPCs is increased by 24 hour after treatment, suggesting early alterations in the transmitter release process similar to those found in degenerating and regenerating nerve terminals. However, morphological studies examining muscles from DTB-treated rats have demonstrated little nerve degeneration even in chronically-treated rats exhibiting marked neuromuscular weakness, although terminal retraction and sprouting are observed (Kemplay, 1984; Jones, 1989).

In conclusion, both quantal content of nerve evoked EPCs and τ_{MEPC} for the normal fast rise and decay MEPCs are decreased in muscles taken from rats exhibiting muscle weakness following chronic treatment with DTB. τ_{MEPC} and τ_{EPC} are both decreased in muscles taken from rats treated 24 hour previously with a single large dose of DTB, while quantal content of evoked EPCs is not depressed significantly. Thus, following exposure to DTB shortening of the decay of synaptic currents is observed, possibly indicating a DTB-induced alteration in the duration

which ACh remains bound to its receptor or a decrease in the open time for AChR-gated ionic channels. These postsynaptic alterations occur prior to presynaptic effects of DTB, which result in a decrease in quantal content of evoked EPCs. Whether these alterations are ultimately directly responsible for this muscle weakness or lead to secondary compensatory changes which result in the observed weakness is not yet clear. These postsynaptic alterations are not likely to be responsible for the early-occurring large amplitude "slow MEPCs" so characteristic of the neuromuscular action of DTB.

Chapter 7. Effects of DTB exposure on single-channel currents.

Summary of results on end-plate currents. Results of the previous voltage clamp experiments indicate that the decay time constant for EPCs and MEPCs is decreased in muscles taken from rats treated with DTB. These results suggest that DTB may act at AChR ion-channels present at the end-plate region to *decrease* the time which channels remain open. However, the EPCs and MEPCs which were studied result from transmitter release from the nerve terminal. Alterations in release processes, synaptic cleft morphology or cholinesterase activity could still alter these currents and thus obscure, potentiate or confound postsynaptic events.

Objective. The purpose of the following study was to determine directly, using single-channel recording techniques, whether the observed decrease in τ_{EPC} and τ_{MEPC} for fast MEPCs is due to an effect of DTB on current flow through nicotinic AChR-channels.

Introduction.

Patch voltage clamp. Application of a low resistance (1 to 10 MΩ), heat-polished, microelectrode to the surface of a cell membrane causes the formation of a seal between the electrode and the cell membrane. Typically the resistance of the seal which forms under these conditions is on the order of 50 to 100 MΩ (Neher and Sakmann, 1976). By applying negative pressure to the interior of the microelectrode

following initial contact with the cell can lead to the formation of a very high resistance seal, on the order of 1 to 100 GΩ, known as a "giga-seal" (Hamill et al., 1981). Due to the restricted area of membrane recorded from and the extremely high electrical resistance of this seal, background noise is decreased to a level at which currents can be observed which are due to the passage of ions through single channels in the membrane (Neher and Sakmann, 1976; Hamill et al., 1981).

The ability to measure current flow at the single channel level allows one to determine accurately whether compounds of interest alter the single-channel conductance or processes associated with opening and closing of channels (Neher and Sakmann, 1976; Hamill and Sakmann, 1981; Colquhoun and Sakmann, 1981). The seal formed following suction is mechanically very stable. This allows several variations on the traditional cell-attached patch configuration described above. First, by applying a second pulse of suction following formation of a giga-seal, or by passing an oscillating voltage across the membrane, the patch of membrane inside the electrode can be ruptured resulting in what is known as the "whole-cell patch" configuration. This configuration allows both chemical access to the interior of the cell and electrical access to the whole cell membrane. Using this technique with small spherical cells allows one to clamp the membrane potential of the entire cell and record currents from the whole cell, similar to those recorded using two microelectrode voltage clamp described earlier. Second, following formation of a giga-seal (cell-attached patch) the electrode can be pulled away from the cell causing formation of a small vesicle at the tip of the electrode. Subsequent movement of the

electrode tip across the air liquid interface leads to rupture of the outer portion of the vesicle. This results in the formation of a patch electrode with a portion of membrane sealed across it, with the intracellular side exposed to the bathing medium. This configuration is referred to as the "inside-out, cell-free" patch. Finally, if one pulls the electrode away from the cell when in the whole-cell configuration a portion of membrane will pull free and form a patch across the electrode tip which has its extracellular side out and is thus known as the "outside-out, cell-free patch" (Hamill et al., 1981). An advantage of the cell-free configuration is the ability to alter constantly or make additions to the solutions to which the exposed portion of the membrane is in contact with. This allows one to examine the effects of different compounds on a single patch of membrane and to examine the reversibility of the observed effects.

Study of Cells in Culture. In the early 1900's Harrison (1907) made the discovery that embryonic neural tissue from frog could be maintained for extended periods of time outside of the organism in a tissue culture environment. The high degree of control offered by cell culture has led to widespread use of this technique in the years since Harrison's original discovery. A wide variety of cells are presently used for cell culture studies, including acutely dispersed embryonic cells (primary cell culture) and transformed cell lines (clonal cells). Primary cell culture offers the advantage of being normal, albeit embryonic cells, taken from some tissue of interest. Thus, the characteristics of these cells should be similar to those of cells within the organism. Problems associated with using primary culture are the

contamination of the culture with other cells (i.e. fibroblasts or glial cells) making it difficult to identify the cell of interest. Second, primary cells arise from embryonic tissue thus, some characteristics of these cells may differ markedly from those of cells in adult organisms. Clonal cell lines, on the other hand, offer a very homogeneous population all with similar or identical characteristics. However, since these cells are transformed cells (neoplastic cells) they can differ in many ways from normal cells, thus, it is not certain how well findings in these cells correlate to those of normal cells.

Rationale for cell culture studies. The use of cells in culture offers several advantages over the use of intact tissues taken from treated animals. First, in a cell culture system we can monitor the effects of DTB on only the cells in question without worrying about toxicity to other systems in the rat. In addition, in cell cultures we can monitor continuously changes in cell behavior following exposure to a set level of DTB without worrying about metabolism and excretion. Access to cells, both pre- and postsynaptic, for both electrophysiological recording and iontophoresis is far easier in cultures due to the lack of connective tissue and surrounding cells. Moreover, little metabolism should occur within our culture systems, thus we can be fairly certain that observed effects are due to the compound added (either DTB or a metabolite thereof).

Clonal G8 myoblast cells. All single channel recording experiments were performed on G8 myoblast cells. G8 cells are a clonal cell line from mouse which, when differentiated to myotubes, express AChR-channels. These cells were obtained

from American Type Culture Collection (Rockville, MD) and were grown on collagen-coated culture dishes in medium consisting of Dulbecco's modified eagles medium containing 10% horse serum and 10% fetal bovine serum. Cells were grown in culture dishes until confluent, at which time the medium was changed to one containing 2% fetal bovine serum and 2% horse serum. Decreasing the serum content of the growth medium in this way enhances differentiation of cells to form multinucleated spindles, which are highly responsive to ACh (Morris et al., 1989). All studies were performed on cells of passage 20-30, however, some differences were noted in cells later than passage 25. Thus care should be taken to use cells around passage 20. Several different ACh-induced channel-conductances have been reported in these cells (Morris and Montpetit, 1986; Morris et al., 1989) and were also observed in the studies reported herein. Channels having a slope conductance of 37 pS predominated. Only this class was used for further analysis.

Methods.

In the following study standard patch voltage clamp techniques were utilized on outside-out, cell-free patches (Hamill et al., 1981) formed from differentiated G8 myotubes. Channel conductance and open and closed durations for channels were determined for single-channel currents recorded from membrane patches of cells grown in DTB for 1-3 days or from cells exposed acutely to DTB in the recording solution.

Results.

Single-channel currents. Figure 28 shows the changes observed in a patch in which the membrane potential was clamped at -100 mV, following a change in the perfusing solution from one containing no agonist to one containing 10 μM SCh_2. In the absence of agonist there is little or no channel activity. Within 1-2 minutes of changing the perfusing solution to one containing agonist single-channel currents can be observed. By 3-4 minutes following addition of SCh_2, as the concentration of agonist in the bath reaches as steady state, a fairly high level of channel activity can be observed. With continued exposure to this relatively high concentration of agonist, desensitization of AChR-channels occurs leading to a decrease in the observed frequency of channel openings (Jackson, 1988). Following desensitization (5 to 10 min) channel openings occur in bursts of single channel activity followed by periods of inactivity. This bursting phenomenon termed "*nachschlag* (second helping)" is thought to represent a single channel returning from an inactive state to an active state, opening and closing several times in quick succession and then returning to an inactive state (Colquhoun and Sakmann, 1981).

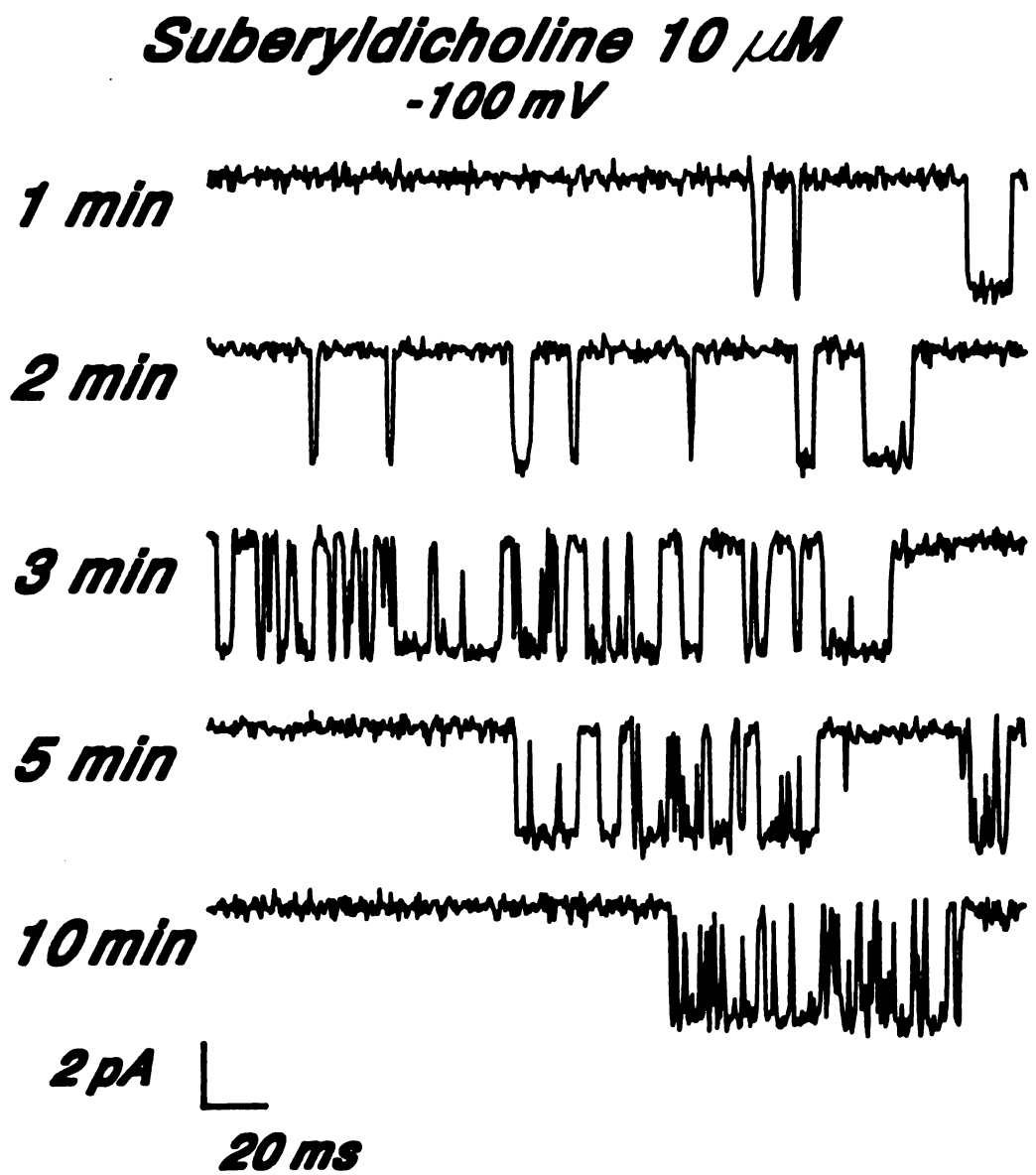


Figure 28. Single-channel currents.

Single channel currents recorded from an outside-out membrane patch at a holding potential of -100 mV. Cell-free patches were formed from G8 myotubes differentiated for 4-6 days in medium containing 2% fetal bovine serum and 2% horse serum. Following patch formation the perfusing solution was changed to one containing 10 μM SCh_2 and currents were recorded for 20 min. Currents shown are filtered at a 2 KHz level.

In the last trace (10 min) many rapid transitions between the open and closed state are observed. This is believed to represent open-channel block of the AChR-channel by agonist. This phenomenon has been observed for all of the different agonist molecules examined thus far and a variety of other positively charged (and some neutral) molecules (Neher, 1983; Ogden and Colquhoun, 1985; Marshall et al., 1991).

Single-channel conductance. A characteristic of the G8 myotubes used in these experiments is the presence of several different conductance classes of channels (Figure 29). These could represent separate channels each with a different conductance, or a single population of channels which show subconductance states of their main conductance level. The majority of channels recorded had the same conductance, which was calculated to be approximately 37 pS. Only these channels were characterized in further studies. If single-channel currents are measured at a variety of holding potentials and the amplitude of the currents plotted vs holding potential, a current vs voltage relationship can be determined for the channels present within the patch. Figure 30 shows current vs voltage relationships generated from patches from 5 control cells and 5 cells exposed for 30 minutes to DTB. The conductance of the channels active in the patches can be determined from the slope of the line, as well as the reversal potential for the channels from the point at which the line crosses the x-axis. From this figure it can be seen that exposure of patches to DTB for 30 minutes appears to have no effect on single channel conductance.

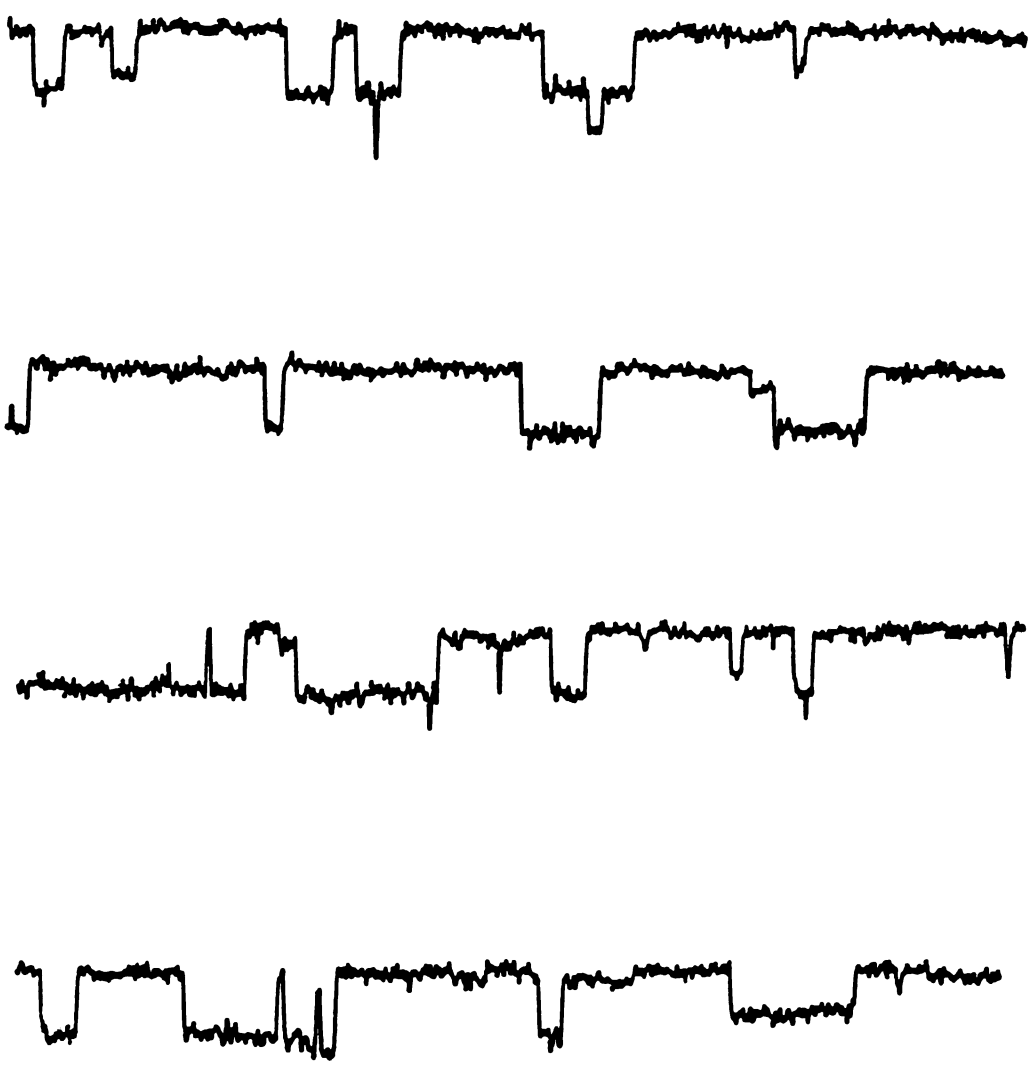


Figure 29. Multiple conductance levels for single-channels.

Multiple conductance levels observed in some patches could represent subconductance states of channels or multiple channel types. Single channel currents elicited by 100 nM ACh were recorded in outside-out patches held at -100 mV. Currents were filtered at a 2 KHz level.

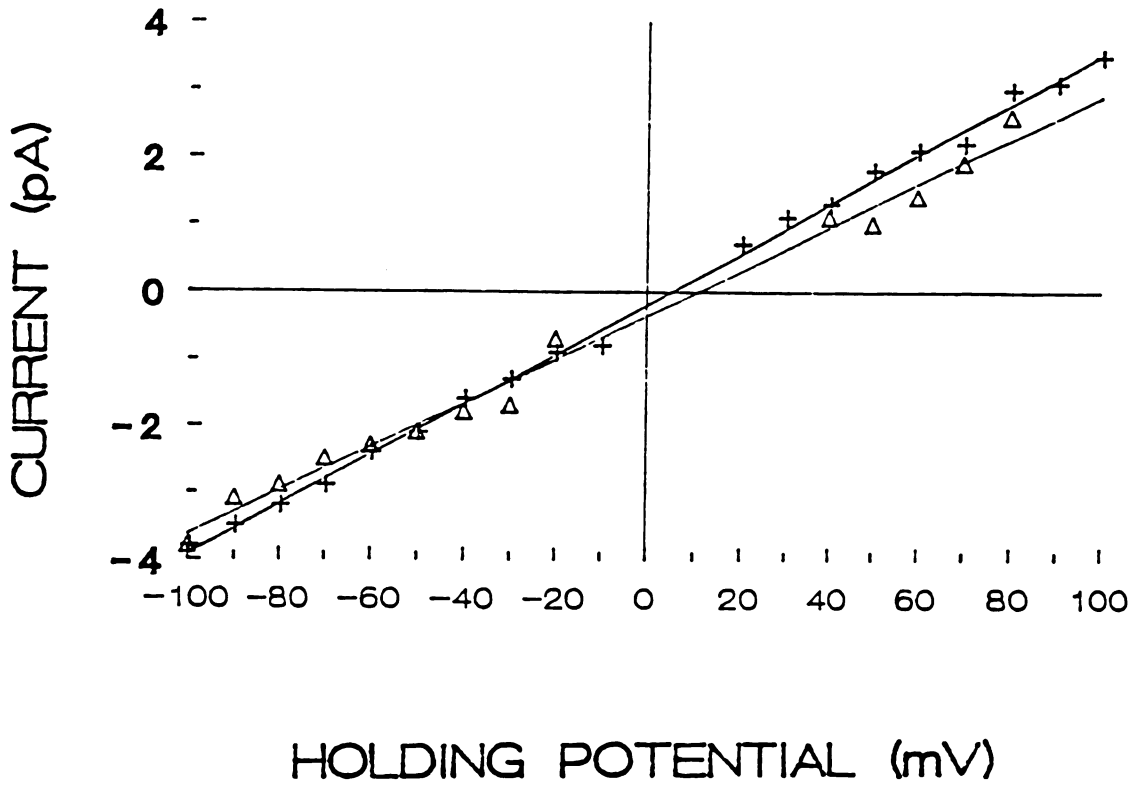


Figure 30. Current vs Voltage relationship for single-channel currents.

Effect of DTB (10 μM) on current vs voltage relationship for single channels. Single channel currents were recorded from outside-out membrane patches at holding potentials between +50 mV to -100 mV in the presence of 100 nM ACh. Currents were recorded in the absence of DTB for 5 minutes then the perfusing solution was changed to one containing 10 μM DTB and currents were recorded continuously for 30 minutes. The data shown represent currents recorded following 30 minutes of exposure to DTB. Only the amplitude of the predominant conductance class present in patches were analyzed. Data shown represent the mean single-channel current of patches excised from cells from 3 different culture-dishes before and after exposure to DTB.

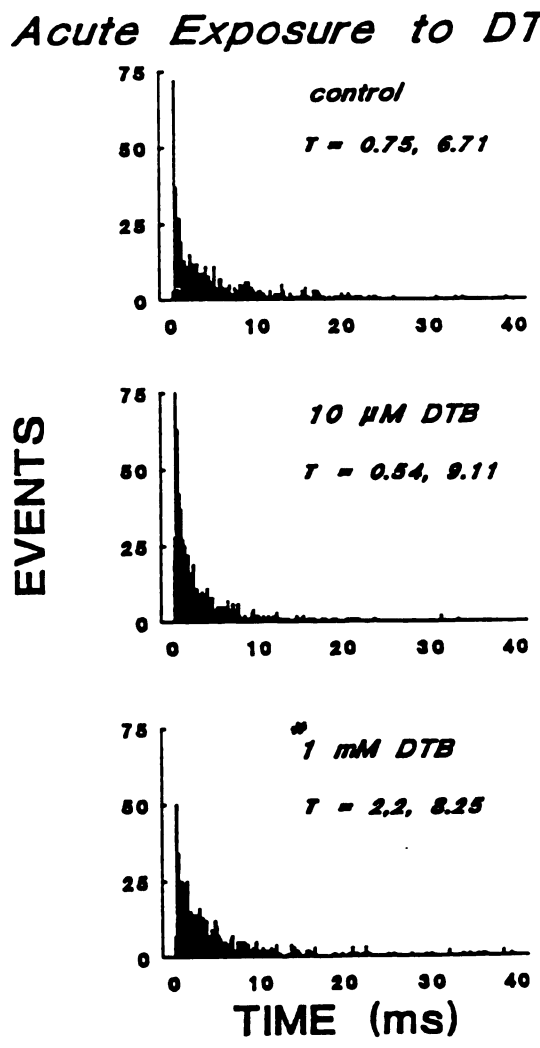
Table 2. Effect of DTB on single channel conductance.

Treatment	*Agonist*	*Amplitude*
Control	ACh	3.34 ± 0.1
30 min 10 μM DTB	ACh	3.21 ± 0.07
30 min 1 mM DTB	ACh	3.26 ± 0.07
Control	SCh_2	3.13 ± 0.06
5 min 1 μM DTB	SCh_2	3.02 ± 0.07
30 min 1 μM DTB	SCh_2	3.02 ± 0.07
# Control	ACh	2.52 ± 0.05
24 hr 1 μM DTB	ACh	2.94 ± 0.25
24 hr 10 μM DTB	ACh	* 3.10 ± 0.15

later passage * different from control $p<.05$

Table 2 displays data recorded from cells exposed to DTB in their growth medium and cells exposed to DTB only during recording. From this it can be seen that exposure of cells to DTB for 24 hour may have an effect on channel conductance, however, these cells were of a later passage and the conductance of control cells had shifted slightly from earlier passages. This makes it difficult to determine whether this is a true increase in conductance or a shift in the type of channels expressed.

Effects of DTB on open and closed durations. Upon examination of a current trace containing single channel events (as in Figure 28) it can be seen that once open, the duration which channels remain open is quite variable. Many long-lived channel openings are interrupted by short closures. If one plots the frequency of channels which remain open for a given duration of time (open-time) vs open-time, a distribution is formed which describes the duration which channels remain open in the patch (open-time distribution). Figure 31 (top) shows an open-time distribution (or histogram) for single-channel currents recorded from a control patch. When this histogram was fit with an exponential curve it was found that the distribution was described by the sum of two exponentials. In Figure 31 center and bottom the effects of exposure to 10 μM and 1 mM DTB for 30 minutes can be seen. Following exposure to 10 μM DTB the biphasic decay of single-channel currents is more pronounced (Figure 31 center). At this concentration of DTB it appears as though the fast time component of open-time (fast τ) is decreased while the slow component (slow τ) is increased or unchanged. Following exposure to 1 mM DTB both the fast and slow components appear prolonged.



* Recorded from same patches as control.

Figure 31. Open-time distributions for single-channel currents.

Open-time distributions from patches excised from control cells and exposed to extracellular medium containing (top) 100 nM ACh, extracellular medium with (center) 100 nM ACh plus 10 μM DTB or extracellular medium containing (bottom) 100 nM ACh and 1 mM DTB. Currents were recorded from outside-out patches at a holding potential of -100 mV. Each distribution represents data pooled from two cells in two different culture dishes. The control and 1 mM data come from the same patches before and 30 minutes after changing the perfusing medium to one containing 1 mM DTB.

Figure 32 shows the effect of exposure to 1 μM DTB on open-time when SCh_2 is used as agonist. SCh_2 binds to the AChR with a greater affinity than ACh and therefore maintains the channel in an open-state for longer periods of time. It was hoped that by prolonging channel open-time by using SCh_2 as agonist, changes in open-time following exposure to DTB would become more evident. Following 30 minutes of exposure to DTB, fast τ is decreased for channels opening in the presence of 100 nM SCh_2, while the slow τ appears unchanged (Figure 32). The duration of the slow events does appear to be decreased following 5 minutes exposure to DTB (Figure 32). When cells are grown in the presence of DTB, thus increasing the duration of exposure to 1-3 days, an effect on channel open-time can be observed when ACh is used as agonist. Figure 33 shows that exposure to DTB (1 and 10 μM) causes a decrease in fast τ, while appearing to prolong slow τ.

When closed-time distributions are examined in a similar manner it is observed that these are also fit by the sum of two exponentials (Figure 34). Exposure to DTB at concentrations between 1 and 1000 μM prolongs the duration of both the short and long closed times. Figure 35 shows the effects of exposure of cells to 1 μM DTB for 24-48 hour on the fast and slow phase of closed durations.

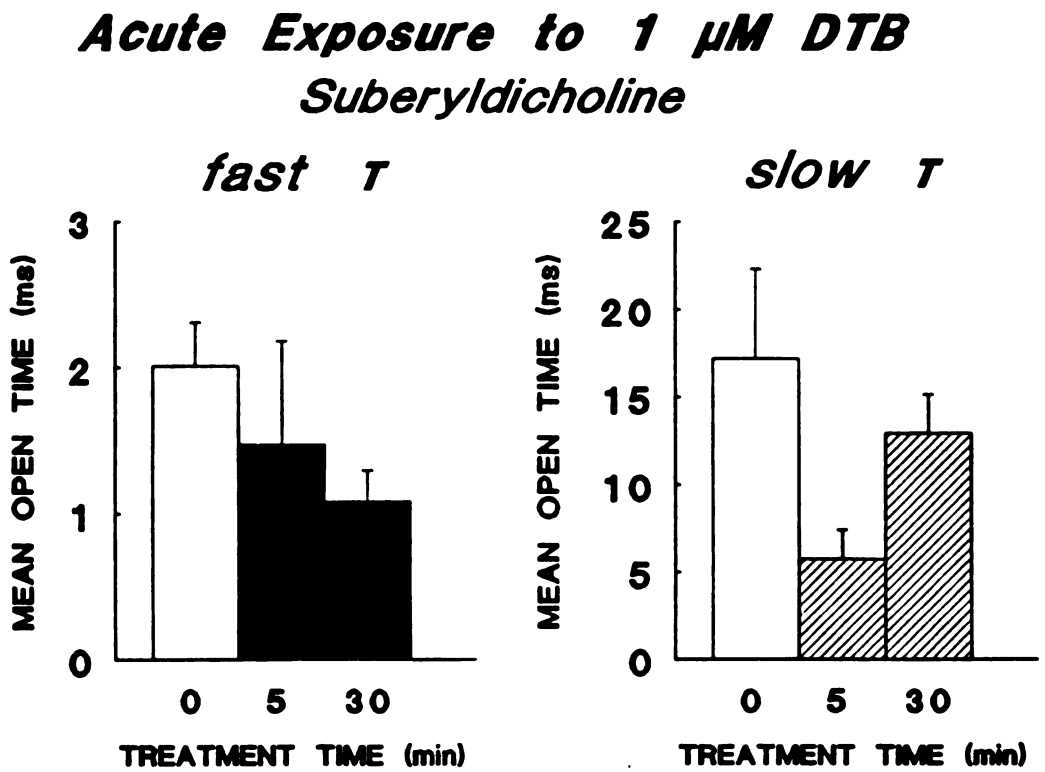


Figure 32. Effect of acute exposure to DTB on single-channel open-time (SCh_2 as agonist).

Effects of exposure to 1 μM DTB on the open-time of single-channel currents recorded in the presence of 100 nM SCh_2. Currents were recorded from outside-out patches held at -100 mV. Open-time histograms were fit with exponential functions which were the sum of two exponentials. The panel on the left shows the effect on the fast τ, while that on the right displays effects on slow τ. Data shown represent the mean ± SEM of currents recorded from patches of control cells from 12 dishes and currents from patches exposed to DTB from cells in 6 dishes.

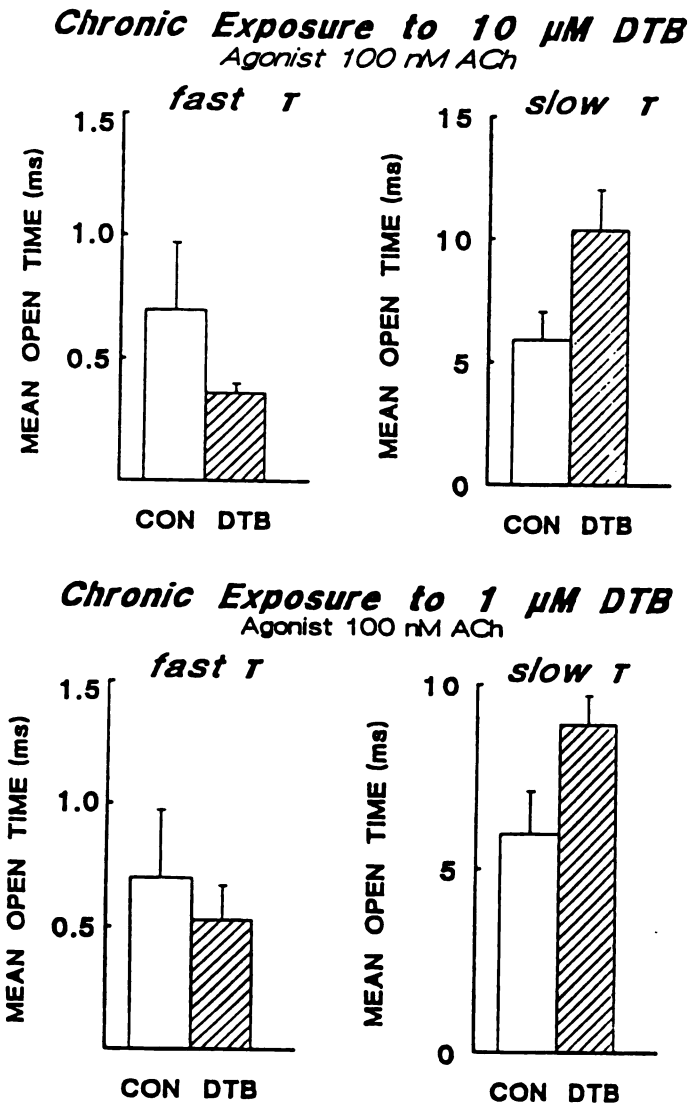


Figure 33. Effect of chronic exposure to DTB on single-channel open-time (ACh as agonist).

Effects of DTB on open-time for AChR-currents recorded from control cells and cells treated with 1 (top) and 10 μM DTB (bottom) for 24-48 hr. Currents were recorded at a holding potential of -100 mV from outside-out patches in the presence of 100 nM ACh. Open-time distributions were fit by a double exponential function. Values represent the mean ± SEM of 1 to 3 cells per plate from at least 4 different plates of cells. The asterisk (*) denotes values significantly different at $p < 0.05$.

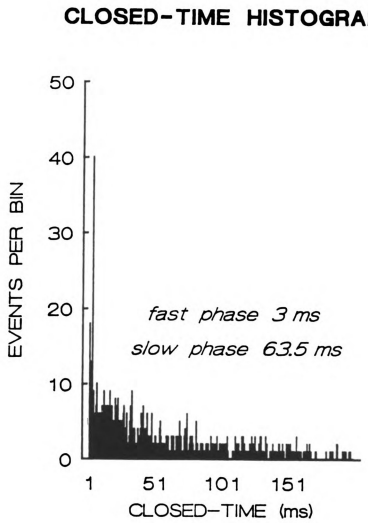


Figure 34. Closed-time distribution for single-channel currents.

Distribution of closed times determined from an outside-out patch from a control cell held at -100 mV in which currents were elicited due to exposure to 100 nM ACh. The distribution of closed times shown here is fit by the sum of two exponentials.

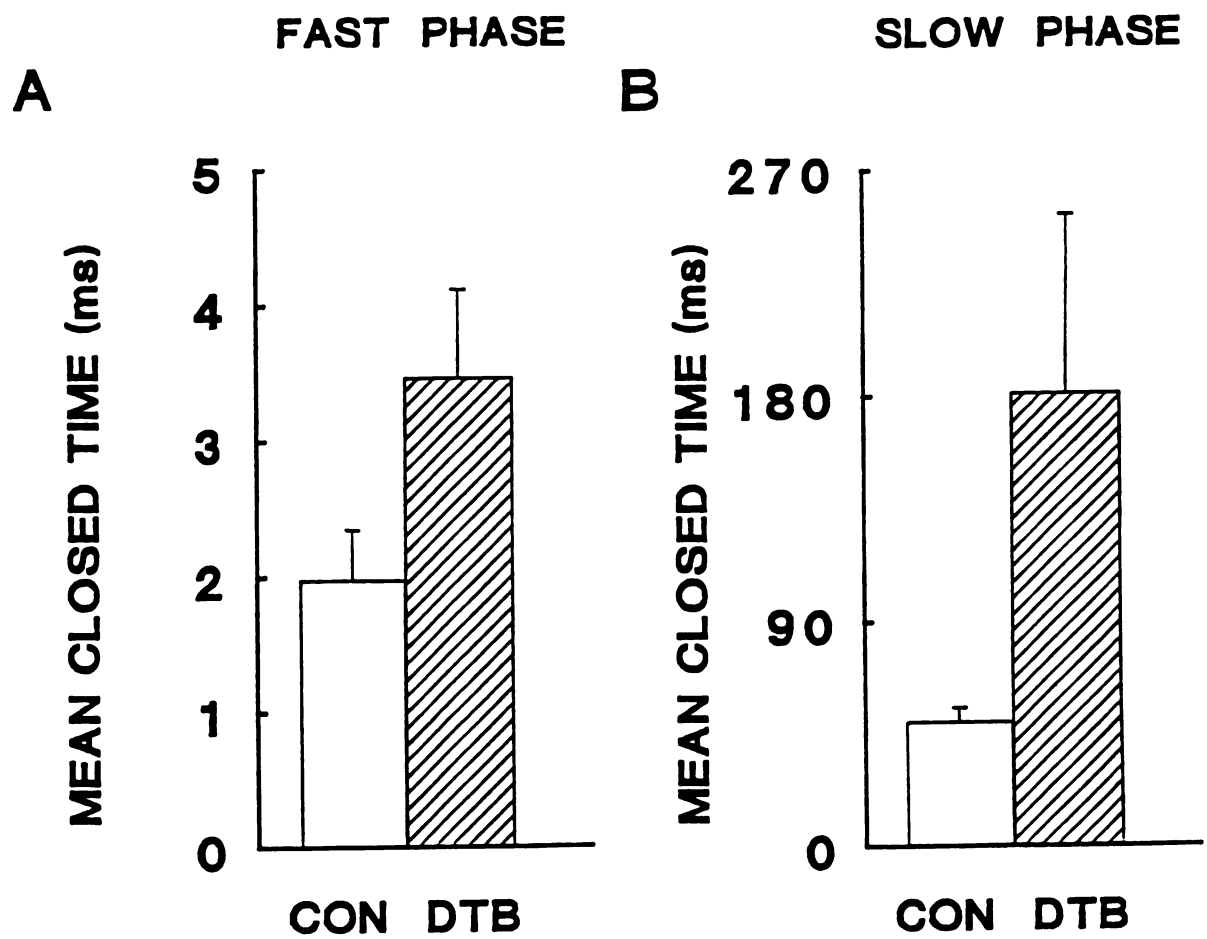


Figure 35. Effect of DTB on the distribution of closed-times.

Effects of DTB on the distribution of closed-times. Channel currents were recorded in the presence of 100 nM ACh from outside-out patches held at -100 mV. Currents were recorded from control cells or cells grown for 24-48 hour in medium containing 1 μM DTB. Closed-time distributions were fit by the sum of two exponentials. Data shown represent the mean ± SEM of data gathered from at least 5 different culture-dishes.

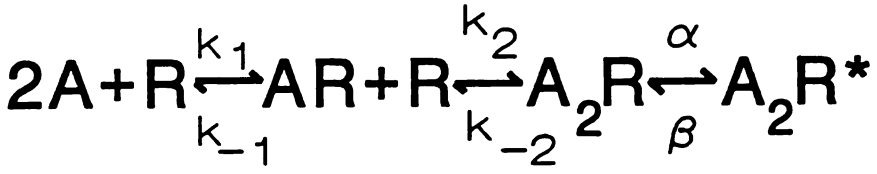
Discussion.

Multiexponential open and closed distributions. Until recently, the model for the binding of ACh to nicotinic AChR-channel complexes and subsequent opening of the associated channel was similar to that shown in Figure 36A (Del Castillo and Katz, 1957). In this model two molecules of ACh (A in Figure 36) bind to the receptor (R in Figure 36), the receptor-channel complex undergoes a conformational change (A_2R to A_2R^* in Figure 36) and this leads to opening of the channel (A_2R^* represents the open AChR-channel complex). After remaining open for a short period of time proportional to α (α is the rate constant for channel closing in Figure 36) the channel closes and agonist molecules dissociate from the receptor-channel complex. It can be demonstrated that the lifetime in any single state (i.e. $2AR^*$ in Figure 36A) is exponentially distributed where:

the mean lifetime of that state = 1 / (sum of transition rates that lead away from the state)

(from Colquhoun and Hawkes, 1985). If the distribution of channel open-times for a channel acting via the model in Figure 36A were constructed, it would be described by a single exponential (since only 1 transition rate leads away from $2AR^*$) and the mean open-time τ for the channels would be equal to $1 / \alpha$ (Colquhoun and Hawkes, 1985).

A



B

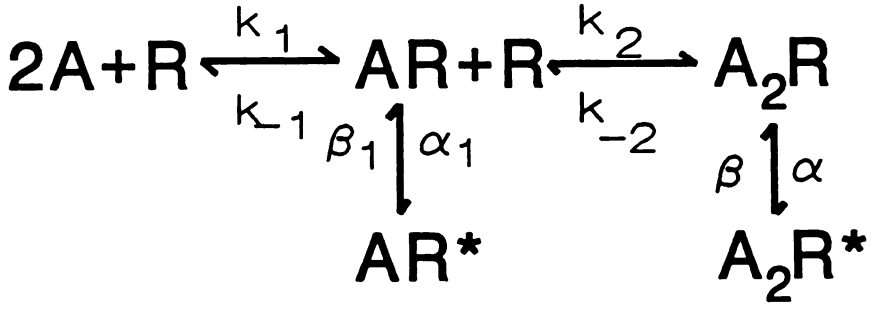


Figure 36. Model of ACh binding and channel opening.

In the experiments described above, open-time distributions, for single-channel events recorded in the presence of 0.1-1 μM ACh or SCh_2, were described by the sum of two exponentials. Biphasic distributions for channel open-time have been reported by several groups examining single-channel currents at low concentrations of agonist (Jackson et al., 1983; Sine and Steinbach, 1984; Colquhoun and Sakmann, 1985). A biphasic distribution for open-times could indicate several things. There may be two populations of channels present which remain open for different durations, the same class of channel may switch between two states one-open for short durations and one open for long durations, or something may be happening to a portion of the channels to cause them to close prematurely (i.e. channel block). Several recent findings support the theory that short duration openings represent the rapid opening of singly-liganded channels (AR in the model in 9a). First, the short openings occur more frequently at low concentrations of agonist (Jackson et al., 1983; Colquhoun and Sakmann, 1985a; Labarca et al., 1985). The ratio of short openings to long is almost always the same for a given concentration of agonist; thus it is unlikely that a second channel type is responsible for these observations. If a second channel type is responsible, then this channel type must always be present in the same ratio in membranes, and patches must contain a large number of active channels (Colquhoun and Sakmann, 1985a). The voltage-dependence of open-time is similar for channels with brief open-times and long open-times indicating that block of the long duration channels (by a charged molecule) is unlikely. The preponderance of data support the theory that the short

duration openings result from activation of a AChR-channel complex by a single molecule of ACh. The relatively high incidence of these short duration openings at high agonist concentrations, when the number of AChRs bound to only one molecule of ACh should be very low, makes it unlikely that all short openings result from singly-liganded receptors (Sine and Steinbach, 1984; Colquhoun and Sakmann, 1985a).

Multiexponential closed-time distributions have also been reported recently (Colquhoun and Sakmann, 1981; Sine and Steinbach, 1984; Colquhoun and Sakmann, 1985a; 1985b), indicating at least 3 different closed states for the AChR-channel complex. Two of the observed closed states are short lived, with time constants of 10 to 70 microsecond for the most rapid phase and 0.1 to 1.2 milliseconds for the intermediate phase, depending on agonist. The third time constant is normally in the range of hundreds of milliseconds (Colquhoun and Sakmann, 1985a). The fast time constants are thought to represent rapid fluctuations between the open and closed state which occur within a burst of activity for a single channel, while the long time constant represents the time which the channel remains closed between bursts. When the number of closures per burst was examined it was found that on average each burst contained approximately 2 fast closures (1.9 ± 0.2) when ACh was used as agonist and approximately 4 fast closures (4.1 ± 0.3) when SCh_2 was used as agonist. The intermediate closures occurred very infrequently and therefore, are not well characterized (Colquhoun and Sakmann, 1985a). In the study described herein, the closed durations were fit by a function

which was the sum of two exponentials. The minimum detectable duration for an event (open or closed) with our analysis system is approximately 100 microseconds. The very fast component described by Colquhoun and Sakmann (1985ab) would not be detected well using our analysis system. Therefore, what is reported as mean channel open-time actually represents "apparent" channel open-time. These apparent openings probably represent channel openings within bursts, where the fast component which we report are closures within a burst and the slow components are closures between bursts. In almost all patches which were recorded from more than one channel was active, which in many case made it difficult or impossible to determine whether closed periods represented those within or between bursts or those between bursts of the same channel or two different channels. Only the apparent open-times could be examined with any reliability.

Figure 36B displays a more recent model which describes binding of agonist and opening of channels for AChR-channels (Jackson et al., 1983; Colquhoun and Sakmann, 1985a; 1985b). Using this model both the short duration openings observed at low concentrations of agonist and the bursting behavior observed as agonist concentrations are increased can be described. In this model the normal long duration channel openings correspond to A_2R^*. Two agonist molecules (A_2) bind to the receptor (R), and the channel opens for a short duration of time (A_2R^* represents the doubly-liganded open channel) proportional to α. Upon closing one or both agonist molecules dissociate from the receptor and the cycle can start over again. When the single channel events are examined using very high resolution data

analysis systems, most openings appear as a burst of channel openings separated by brief closures. It appears as though each burst of openings represents an oscillation between the closed A_2R state and the open A_2R^* state, in which the channel opens, closes and reopens before the agonist molecule can dissociate. The results of Colquhoun and Sakmann (1985a) indicate that, on average, each long duration opening observed contains 2 rapid closures when ACh is agonist and 4 rapid closures when SCh_2 is agonist. This model also can account for openings associated with the singly-liganded AChR-channel (AR^*). These are normally observed at a low frequency due to the positive cooperativity of binding of agonist to the AChR. The AChR has a greater affinity (up to 100X) for binding of the second agonist molecule once one molecule is bound (Sine et al., 1990).

Effects of DTB on single AChR-channels. The results described above demonstrate that exposure of AChR-channels to low concentrations of DTB (1-10 μM) alters the time which the channels remain open. When ACh is used as agonist (0.1 to 1 μM) it appears as though channels which remain open for short periods of time (fast τ) close faster in the presence of DTB and channels which normally remain open for longer periods of time (slow τ), appear to have prolonged open-times. When the effects of DTB are examined on single-channels observed when SCh_2 (100 nM) is used as agonist, DTB induced changes appear somewhat different. Following exposure of patches to DTB (1-10 μM) for 30 minutes there is a decrease in fast τ but no effect on slow τ. Five minutes after exposure to DTB there appears to be a decrease in slow τ but no change in fast τ. The effects of DTB on channel

open-time become less complicated when examined in a slightly different manner. When determining fast and slow τ from the observed distributions several criteria are used to determine whether the distribution is fit by a single exponential or the sum of multiple exponentials. The x^2 statistic can be used as one criterion. This statistic determines how well the observed data points fit the generated curve. A goodness of fit ratio is also determined by the curve fitting routine when calculating exponential functions to fit the data. This ratio gives information on how many iterations are necessary to obtain a curve which describes the data. Perhaps the best criterion for determining how well an exponential function describes a distribution is to examine by eye how well the generated function appears to fit the actual data (Colquhoun and Sigworth, 1985). In many patches it was difficult to determine whether the distribution for channel open-time was fit better by a single exponential or the sum of two exponentials and data from several patches were definitely fit best by a single exponential. When the effects of DTB on open-time are examined in distributions which are fit by a single exponential the changes observed become much more consistent. Figure 37 displays the effects of DTB (1 μM) on channel open-time when all distributions were fit with single exponentials. When examined in this manner a decrease in apparent channel open-time is observed following exposure of cells or patches to DTB.

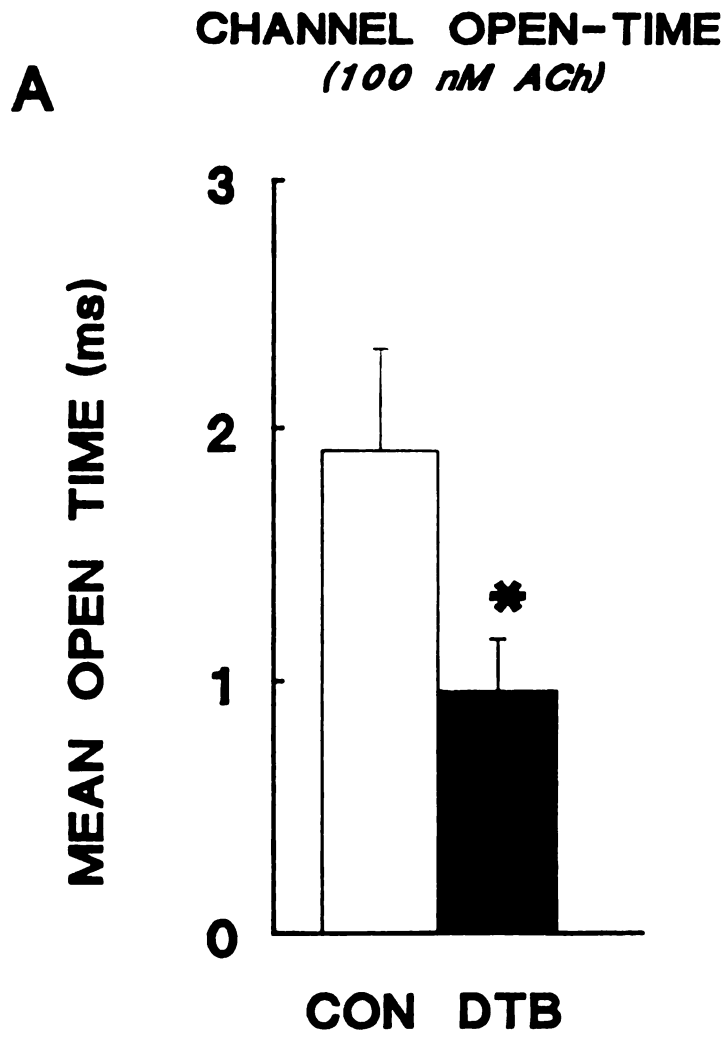


Figure 37. Effect of DTB on single-channel open-time (single exponential fit).

Effect of DTB on channel open-time when open-time distributions are fit with a single exponential function. Single channel currents were recorded as described in Figure 33, however, the distributions of open-times were fit with a single exponential function instead of the sum of two exponentials. Values shown represent the mean ± SEM of patches from at least 5 culture-dishes.

Results of these studies indicate that DTB has a direct effect on single channels to decrease the time which they remain open. This effect of DTB could explain the observations in previous voltage clamp experiments in which τ_{EPC} and τ_{MEPC} were found to be decreased in muscles from rats treated with DTB.

DTB could interact with the AChR-channel complex in several ways to cause the observed decrease in open-time.

Block of open channels by DTB. Many compounds have been found to block the AChR-channel following its opening. All of the agonist molecules tested thus far have been found to block the channel (Ogden and Colquhoun, 1983; Sine and Steinbach, 1984), moreover, agents such as d-tubocurarine, thought of as classical receptor blockers, also block the ion channel (Colquhoun et al., 1979). In addition, a variety of other charged and uncharged molecules (Neher and Steinbach, 1978; Ogden et al., 1981; Neher, 1983) also appear to block the channel physically. During open-channel block, effects on both macroscopic (i.e. EPCs and MEPCs) and single-channel currents can be observed which are very characteristic for this mechanism. In the presence of compounds which can block open channels the single-channel currents exhibit very characteristic bursts of activity. These bursts (Figure 28 last panel) represent a single channel opening and becoming blocked and unblocked in rapid succession, followed by channel closure. The effects on EPCs and MEPCs of open-channel block are observed as a biphasic decay of the current. In the presence of open-channel blockers the normal number of ion-channels open following release of transmitter into the synaptic cleft, thus the initial

amplitude of the current is normal. Soon after opening 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 prior to their final closure (Adams and Sakmann, 1978; Lambert et al., 1980). When the effects of DTB on channel opening are examined neither the characteristic bursting of single channel currents, nor biphasic decay of EPCs or MEPCs is observed. This probably indicates that DTB does not block the open channels. In addition, a plot of $\tau_f^{-1} + \tau_s^{-1} - \tau_c^{-1}$ vs concentration of DTB will be linear if DTB is blocking the open channels (where; τ_f = fast τ and τ_s = slow τ, in the presence of DTB and τ_c is the single control τ) (Ogden et al., 1981). Figure 38 shows a plot of $\tau_f^{-1} + \tau_s^{-1} - \tau_c^{-1}$ vs concentration of DTB for single-channel currents measured in the presence of 100 nM ACh. This plot is not linear, thus, DTB does not appear to act via open-channel block to decrease the open-time of single-channel currents.

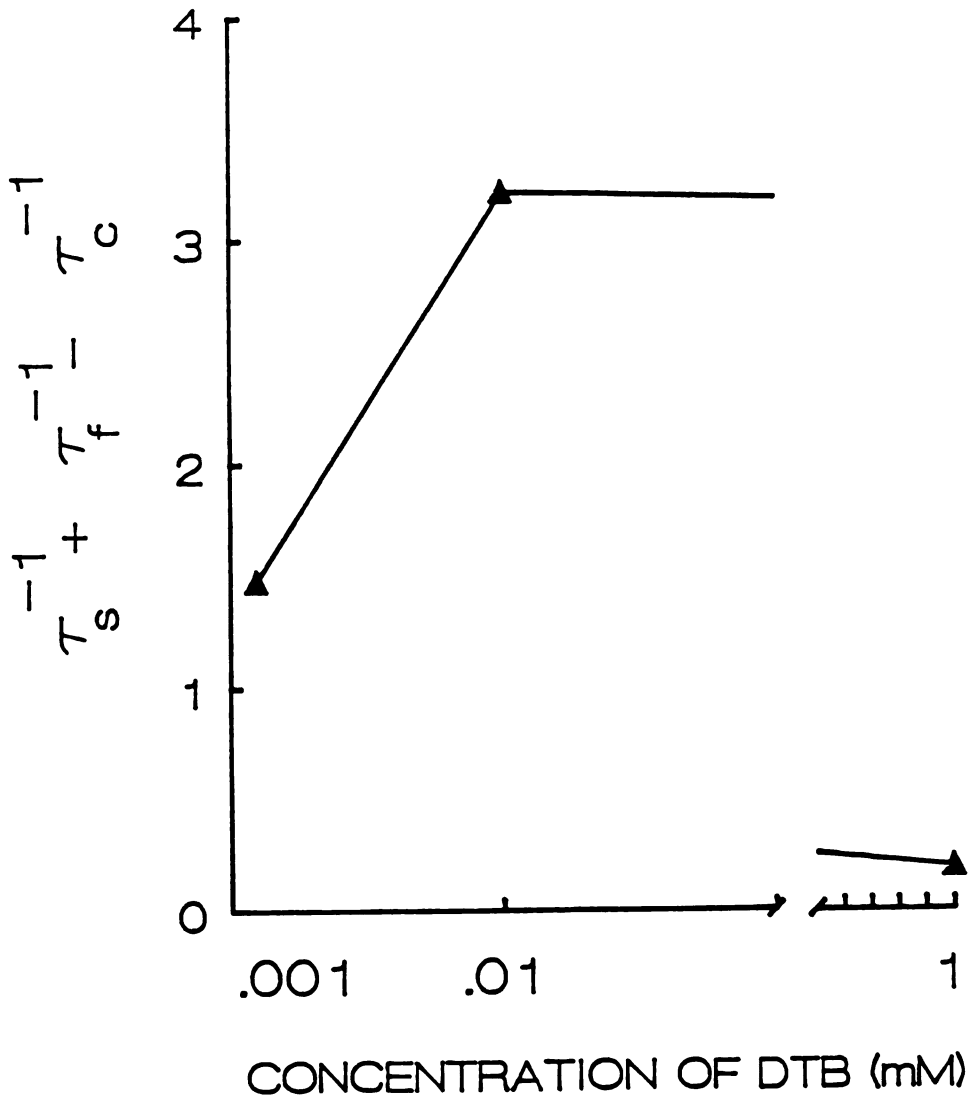


Figure 38. Plot of $\tau_f^{-1} + \tau_s^{-1} - \tau_c^{-1}$ vs concentration of DTB.

Plot of $\tau_f^{-1} + \tau_s^{-1} - \tau_c^{-1}$ vs concentration of DTB where; τ_f = fast τ and τ_s = slow τ, in the presence of DTB and τ_c is the single control τ. The data graphed represent open-times for single channels recorded in outside-out patches held at -100 mV with 100 nM ACh as agonist. Channel currents were recorded in the absence and presence of 1-1000 μM DTB. Linearity in this plot would indicate that DTB is affecting channels via open-channel block.

Effects on the affinity of receptors for agonist. Previous studies have demonstrated the presence of disulfide bonds within the receptor region of the AChR-channel complex which are critical to normal function (Karlin and Winnik, 1968; Rang and Ritter, 1971). Reduction of disulfides present on the receptor, with the reducing agent dithiothreitol (DTT), decreases the responsiveness to ACh while reoxidation, with the oxidizing agent 5,5'-dithio-bis (2-nitrobenzoate) (DTNB), reverses these effects when examined in 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 Torpedo electroplax. They observed that reduction of the receptors with DTT decreased the binding affinity of AChRs for carbamylcholine (CCh) and shifted the dose-response curve for CCh-induced increases in $^{22}Na^{+}$ 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 ^{86}Rb influx into vesicles with reconstituted AChR-channels purified from Torpedo californica were measured before and after reduction with DTT. Results of these studies demonstrated that the main effect of DTT reduction was to shift EC_{50} 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. More recently Bernstein et al. (1988) examined the effects of DTT and DTNB on muscarinic AChRs purified from pig brain. They observed that in the presence of DTT the affinity for muscarinic ligands was decreased without

altering the total number of binding sites. Electrophysiological studies performed on muscles exposed to DTT have demonstrated that following 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 following reduction of AChRs with DTT, the time which single-channels remain open and the conductance for these channels are decreased. Results of the above studies indicate that 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 effects of DTB, 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 those observed following reduction with DTT.

Mechanism for a decrease in affinity decreasing open-time. In the model (Figure 36b) there are 2 steps for agonist binding with forward and reverse rate constants of k_1, k_{-1}, k_2 and k_{-2}. The dissociation constants for these two binding steps would be $K_{d1} = k_{-1}/k_1$ and $K_{d2} = k_{-2}/k_2$. In normal channels the majority of openings are going to result from AR binding a second agonist molecule to become A_2R; this combination will open giving A_2R^*. During the average opening there will be two brief closures (Colquhoun and Sakmann, 1985a), in which the AChR-channel oscillates between A_2R^* and A_2R. During the third brief closure (on average) the agonist molecule dissociates from the receptor and it remains shut. When a higher affinity agonist is used, it binds tightly enough to the receptor that

the channel is able to oscillate through 4 brief closures prior to dissociation of the agonist (Colquhoun and Sakmann, 1985a). Following reduction of disulfides located on the receptor the affinity for agonist decreases (K_d increases) (Walker et al., 1981). If the rate of dissociation increases, then the agonist molecule may dissociate during the first or second brief closure, thus decreasing the time that the average channel remains open.

The effects on closed time following DTB exposure complicate this mechanism to a certain extent. The prolongation of the long closed durations fits this scheme very well. If the long closed durations represent closures between bursts when both agonist molecules dissociate from the receptor, then these could be expected to be prolonged if the affinity of the receptor for agonist is decreased (i.e. fewer bindings and openings per unit time). If the short bursts represent rapid transitions between the A_2R^* state and the A_2R state, then a change in affinity should have no effect on this duration. An increase in the duration of short closures is observed in this study. One possibility which could explain this is that brief closures represent transitions between the A_2R^* state and the AR state. In this case changes in affinity would alter both the duration of the short and long closures as well as open-times.

Conclusion. The decrease in channel open-time observed in this study is consistent with a direct effect of DTB on the AChR-channel complex. The decrease in τ_{MEPC} for fast MEPCs observed in earlier studies could be explained by such an effect. It is possible that by reducing critical disulfide groups on the AChR-channel

complex DTB decreases the affinity for binding of agonist to the receptor, which then leads to the observed decrease in open-time.

Chapter 8. Effects of DTB on single-channel current measured via fluctuation analysis.

Summary of DTBs effects on single-channel currents. Results of the previous patch voltage clamp studies indicate that exposure of cultured muscle cells to DTB, at concentrations approximating those measured in the plasma of rats treated chronically (1 mg/kg/day) with DTB (Porter et al., 1983), leads to a decrease in the apparent open-time for nicotinic AChR-channels. Due to the fact that these studies were performed on muscle cells grown in culture in the absence of any presynaptic input, the observed results represent an action of DTB on the AChR-channel itself. The limited area of membrane recorded from and the high resistance of the seals formed make it unlikely that the effects observed represent changes in cable properties of the membrane or ineffective space-clamp of the membrane under study. The purpose of the following study was to determine whether channel open-time is similarly affected in intact preparations taken from rats exhibiting no signs of muscle weakness 24 hour following a single 1 mg/kg dose of DTB or from rats exhibiting muscle weakness following 6-7 days of treatment with DTB (1 mg/kg/day). The technique used measures channel activity in the presence of exogenously applied agonist. Therefore, alterations in presynaptic structure or function should not influence the observed results.

Introduction to fluctuation analysis. The patch voltage clamp technique has proven to be extremely valuable in describing the kinetics of channel opening and closing in a variety of cell types (Hamill et al., 1981; Neher, 1983; Sine and Steinbach, 1984). Patch voltage clamp techniques are limited by the fact that the membrane surface to be patched must be readily accessible and relatively free of contaminating material, such as connective tissue or glial cell processes. Due to these constraints, channel-kinetics in a variety of preparations, such as intact neuromuscular junctions, are difficult to study using this technique. One method used quite frequently to study the behavior of channels at the neuromuscular junction is to cut the nerve to the muscle of interest and to patch clamp the muscle once the nerve has degenerated (Neher and Sakmann, 1976; Reiser and Miledi, 1989). Following denervation there is a change in the number and type of receptor channel-complexes present on the surface of the muscle which makes it difficult to correlate any observed behavior with the channels originally present (Axelsson and Thesleff, 1959; Katz and Miledi, 1973; Neher and Sakmann, 1976). An alternate technique which has been very useful in analyzing channel kinetics in intact preparations is fluctuation analysis. Fluctuation analysis or "noise analysis" as it is sometimes called, is a technique by which both single-channel conductance and mean open time for channels are determined from spontaneous, random variations in membrane noise observed during the opening and closing of channels (Katz and Miledi, 1972; Anderson and Stevens, 1973; Stevens, 1975). This technique was first used to characterize the AChR-gated ion-channel in the early 1970s by Katz and Miledi

(1970; 1972; 1973). They observed that in the presence of a constant application of ACh to frog end-plate regions there was an increase in the noisiness of the recording. By analyzing spectra generated from the random fluctuations in noise they could gain information about the opening and closing of channels underlying the observed noise.

Determination of mean channel open-time with fluctuation analysis. An electrical signal which varies with time (time-varying signal) can be characterized by its frequency spectrum. Analysis of the frequency components of a waveform is known as *fourier analysis*. According to Fourier's theorem, time-varying signals of the type most commonly encountered can be decomposed into a sum of sine and cosine waves of various frequencies. By performing fourier analysis, a time-varying signal is transformed into its spectrum, which is the amplitude of each frequency component in the signal plotted against frequency. Spectra generated using this analysis technique are normally displayed as the power density spectrum, which is the power of each frequency component (watts/Hz) plotted against the frequency (Hz). When displaying spectra it is common to plot the log power vs log frequency to compress the axis and allow one to display information over many orders of magnitude on a single figure. The different spectral components present in the signal can be determined in several ways. The original method used was to record a segment of signal containing data of interest, to filter this signal sequentially with narrow band-pass filters, and to measure the power of the component which is allowed through the filter. By changing the frequency which is allowed to pass

through the filter, all components of the signal can be measured. Presently, the preferred procedure is to use digital computers and an algorithm known as the Fast Fourier Transform (FFT). In order to analyze data on digital computers the signal of interest must be converted from a continuous signal to a digitally sampled one via an analog to digital converter. To be certain that all frequencies of interest are retained, and contaminating frequencies not of interest removed, several things must be accounted for during the process of digitization. First, care must be taken when sampling to record long enough samples of signal to insure that low frequency components of the signal are included. For example, the lowest frequency component present in a 10 sec long recording will be 0.1 Hz. Second, the signal must be sampled at a high enough rate to define high frequency components contained within the signal. The Nyquist sampling theorem provides a quantitative basis for the rate at which a signal must be sampled to insure that all components of interest are retained in the digitized signal. This theorem states that if a band-limited signal is sampled at twice the highest frequency component (Nyquist frequency), the sample values will exactly describe the original signal (Malmstadt et al., 1981). Thus, a signal with frequency components up to 100 Hz should be sampled at twice this rate or 200 Hz, a rate which corresponds to 5 milliseconds per data point. In addition, it is important to filter the signal of interest prior to digitization to remove unwanted components in the high frequency regions. If high frequency components are not removed from the signal an error known as aliasing can occur. When aliasing occurs, high frequency components above the Nyquist

frequency are undersampled and are recorded as spurious low frequency components in the digitized signal. A good rule to follow is to use a low-pass filter (removes high frequency components) with a cutoff frequency equal to one half that of the sampling rate. If a signal of interest is to be sampled at 1 KHz, then this signal should be filtered using a filter which has a cutoff frequency of 500 Hz. This removes frequency components from the signal, prior to sampling, which would lie outside of the spectra to be generated.

Determination of channel amplitude from fluctuations in noise. The single channel conductance can be calculated by measuring the amplitude of the fluctuations in mean current level and comparing these to the amplitude of the mean macroscopic current. The maximum value (power) of the generated spectra should be related to the amplitude of the unitary changes. Thus, both the standard deviation of the noise and the maximum power of the spectra can be used to calculate the conductance of the single channels.

Iontophoresis. Using iontophoresis, charged molecules can be ejected from a high resistance electrode (100-200 MΩ resistance) by passing a constant ejection current through the electrode. A low level braking current, of opposite polarity from the ejection current, must be applied to the electrode when not ejecting drug to prevent leakage from the tip of the electrode and hence receptor desensitization. The amount of compound ejected over time can be estimated from the formula:

$$N = F * I * k * t$$

where: N equals the number of molecules released, F equals Faraday's constant, I is the current passed through the pipet, k is the transport constant of the pipet (approx. 0.25 for ACh ejected from a pipet of 100 megohm resistance) and t is the time of release (Dreyer et al., 1978; Dionne et al., 1978; Warner and Bate, 1987). To record fast voltage or current changes with this procedure the iontophoretic pipet should be brought to within 15-20 μm of the end-plate. For noise analysis better results are obtained if the iontophoretic pipet is placed approximately 100 μm from the end-plate. Increasing the distance between the iontophoretic electrode and the end-plate region dampen out rapid fluctuations in ACh concentration preventing rapid fluctuations in macroscopic currents.

Experimental methods. The experimental design and methods used are described in detail in the materials and methods of this manuscript. Briefly, hemidiaphragm preparations were removed from control rats, rats treated for 1 day with a single 1 mg/kg dose of DTB and from rats exhibiting muscle weakness following chronic treatment with 1 mg/kg/day of DTB. In addition, several muscles taken from control rats were exposed to 1 μM DTB for up to 4 h in the bathing medium. End-plate regions were clamped, using the two microelectrode voltage clamp described earlier. A third microelectrode, containing 2 M ACh, was brought into the region of the end-plate and ACh forced from the microelectrode by passing positive current out of the ACh containing microelectrode (iontophoresis). For these experiments end-plate current was recorded at two different amplification levels. The first, a DC coupled channel, was amplified from 1-10X. This signal allows the

magnitude of the iontophoretic current to be determined. The second, a high amplification (100-1000X), AC coupled, recording allows the random current fluctuations due to the opening and closing of ACh gated ion channels to be characterized. The 100-1000X signal should also be high- and low-pass filtered prior to analysis to remove unwanted components from the spectra.

Results.

Early effects of DTB on MEPCs. Several different populations of MEPCs, some with extremely altered amplitudes and rise and decay kinetics, have been observed at end-plate regions of muscles taken from rats exhibiting muscle weakness following chronic treatment with DTB (see results in Chapter 6). No electrophysiological studies have been performed on muscles from rats treated for 1 day with 1 mg/kg/day of DTB (the daily dose administered during chronic treatment). It would be of interest to determine whether MEPCs are altered at end-plate regions of these muscles. Recordings from these muscles indicate that as early as 1 day following a single 1 mg/kg dose, the frequency of abnormal MEPCs is increased. When control muscles are exposed to 1 μM DTB in the bathing medium for 4 hour the incidence of abnormal MEPCs is also increased (Figure 39).

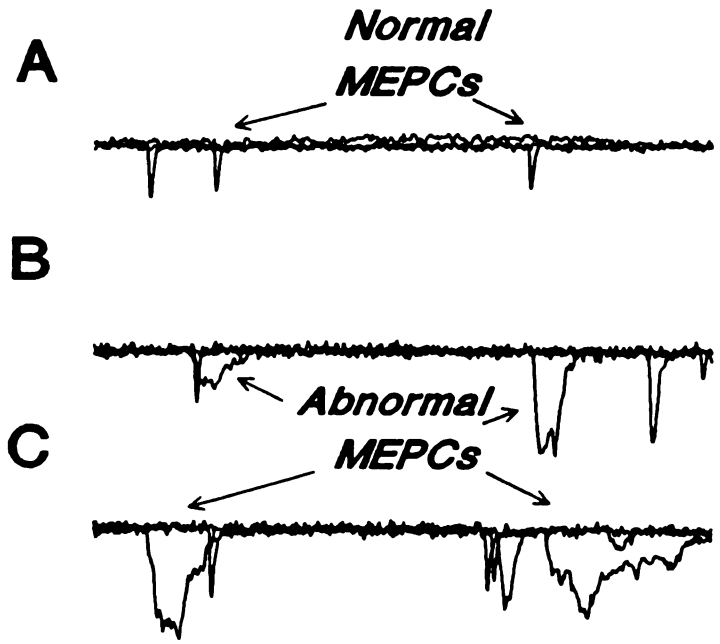


Figure 39. Exposure to DTB causes an increase in the incidence of abnormal MEPCs.

Hemidiaphragm preparations were prepared as in Figure 20 and MEPCs recorded at a holding potential of -70 mV. MEPCs were recorded from neuromuscular preparations taken from A) control rats, B) rats treated 24 hours previously with a single 1 mg/kg dose of DTB and C) from control rats and exposed to 1 μM DTB in the bathing medium.

Fluctuations in iontophoretic current. Following a change in the current flowing through the iontophoretic electrode from -4 pA to +10 pA a current can be measured at the end-plate region which has an amplitude on the order of 5-50 nA (Figure 40). If there are 10^6 channels present at the end-plate region and 10^3 of these open during iontophoresis of ACh, then probability of a single channel being open will be $P = 10^3/10^6 = 0.001$. Thus, the standard deviation of the number of open channels is given by $[NP(1-P)]^{1/2} = 31.6$. The number of channels which will be open at equilibrium, during iontophoresis, will not be constant but will = 1000 ± 31.6 (from Colquhoun and Hawkes, 1985). A fluctuation in the steady current will be observed which represents the opening and closing of approximately 30 channels in the case above. This fluctuation in channel opening can be observed as an increase in the variance in the current level (Figure 41A) and as an increase in the level of noise observed (Figure 41B) within the actual signal.

Determination of the decay time constant τ. Following transformation of the signal, using a fast fourier transform, a power spectrum can be generated (Figure 42). From this it can be seen that the frequencies of noise which contain the most power are in the range of 1 to 100 Hz, while higher frequency components have lower and lower power levels. This power spectrum can then be fit with a Lorentzian function, similar to the fit of single-channel events with exponential functions. This allows several characteristics of the events underlying the current fluctuations to be determined. The decay time constant τ for channels generating the noise can be determined from the corner frequency (f_c) of the spectra (or Lorentzian function).

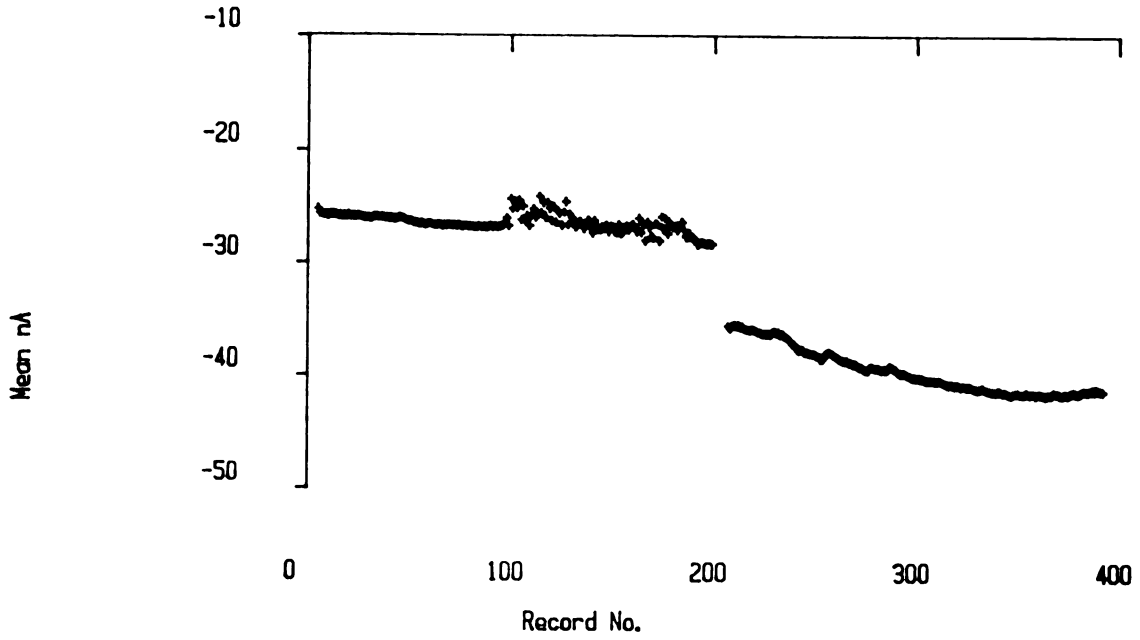


Figure 40. Amplitude and duration of iontophoretic current.

Amplitude and duration of current induced following iontophoretic application of ACh. The membrane potential of end-plate regions was clamped at a constant holding potential of -70 mV using two microelectrode voltage clamp. A third electrode containing 2 molar ACh was placed in the region of the end-plate and an iontophoretic current was elicited by passing positive current through the ACh containing electrode. Each record number on the x-axis represents 250 microseconds.

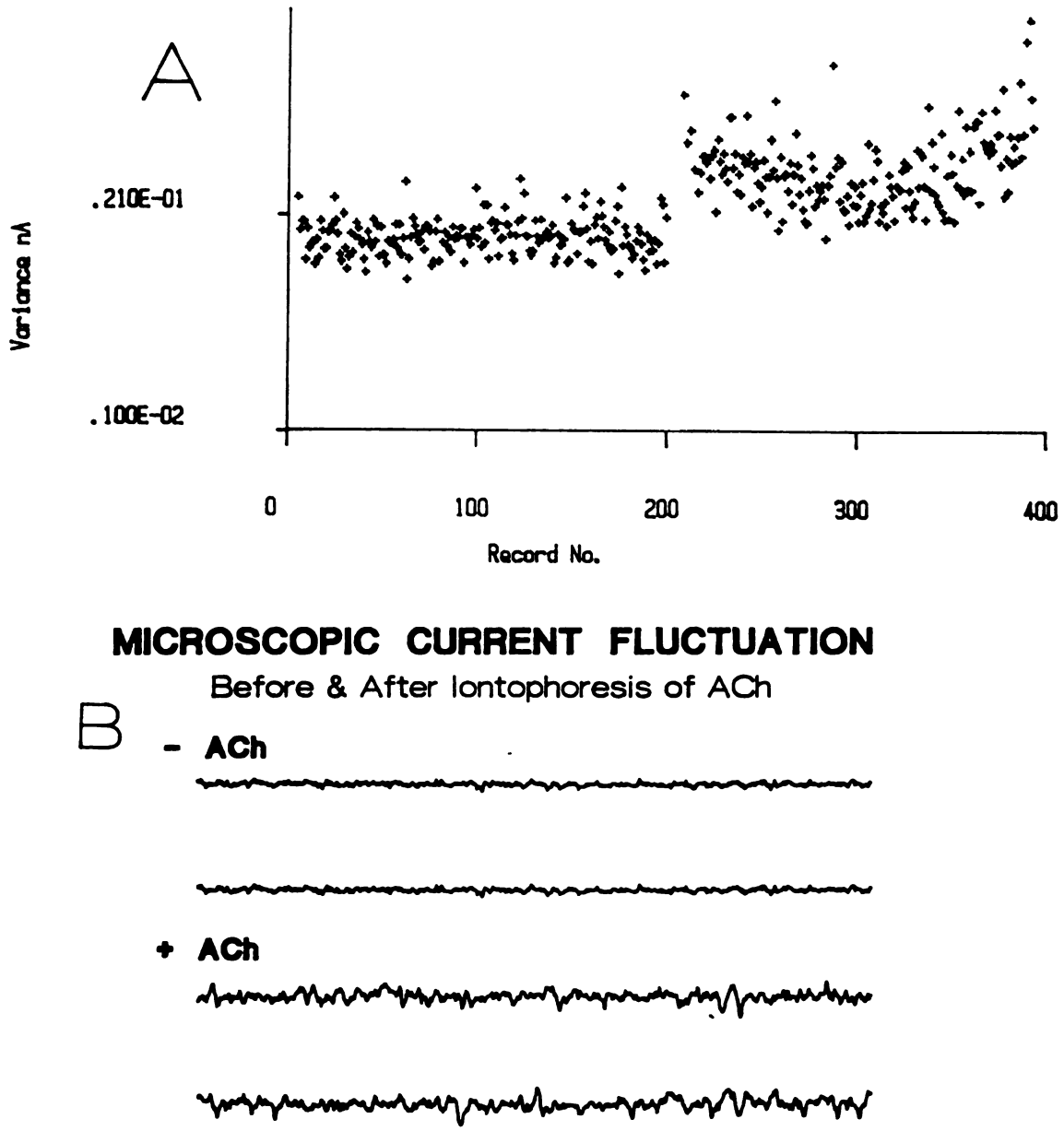


Figure 41. Membrane noise observed during iontophoresis of ACh.

Microscopic fluctuations in current observed during iontophoresis of ACh to end-plate regions. Panel A displays the variance in the microscopic current fluctuations observed during the ACh evoked current shown in Figure 40, each record represents 250 microseconds. Panel B displays membrane noise observed before and during ACh evoked current. Currents were amplified 100X and band-pass filtered from 1-500 Hz.

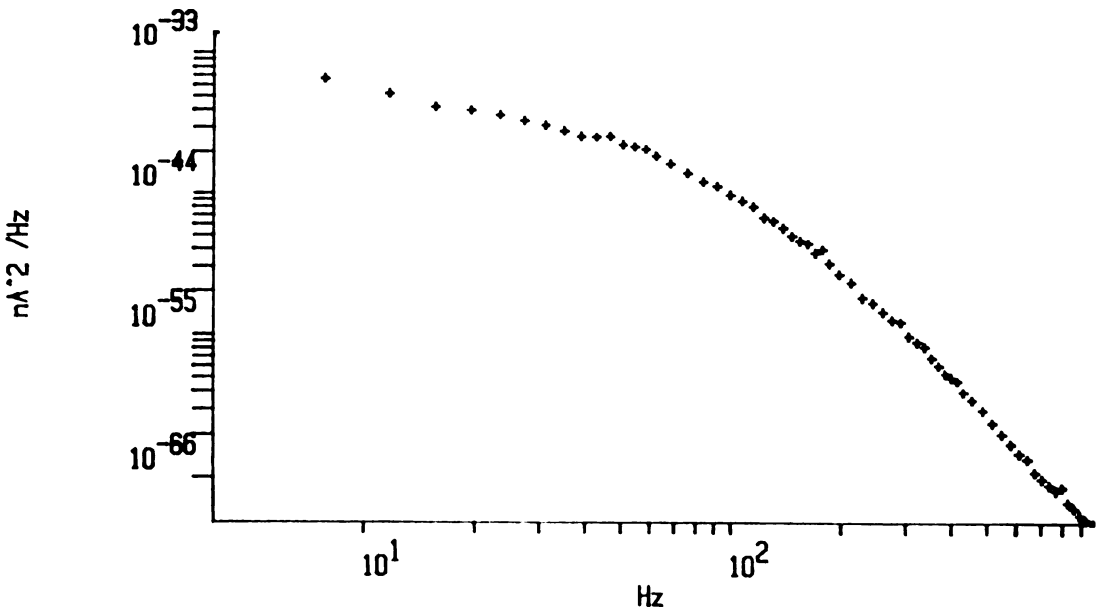


Figure 42. Power spectrum from ACh induced membrane noise.

Power spectrum generated from fluctuations in current recorded during application of ACh to end-plate regions. The membrane potential of end-plate regions was clamped at -70 mV and current was recorded before and after application of ACh. Current signals were amplified 100X and band-pass filtered between 1-500 Hz. Power spectra were generated by fast fourier transform of the recorded signal.

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 τ can be calculated using the equation $\tau = 1/2\pi f_c$ (Anderson and Stevens, 1973). Figure 43 shows values of τ determined for end-plate regions, clamped at potentials between -20 and -70 mV, of muscles taken from control rats. When muscles are taken from rats exhibiting muscle weakness following chronic treatment with DTB, values of τ calculated from spectra are decreased compared to control values (Figure 44A). Values of τ are also decreased in muscles taken following a single dose of DTB indicating that channel open-time is affected before muscle weakness is observed (Figure 44A). τ is also decreased in muscles from control rats exposed to 1 μM DTB in the bathing medium for 4 hours when measured at a holding potential of -70 mV (Figure 44B).

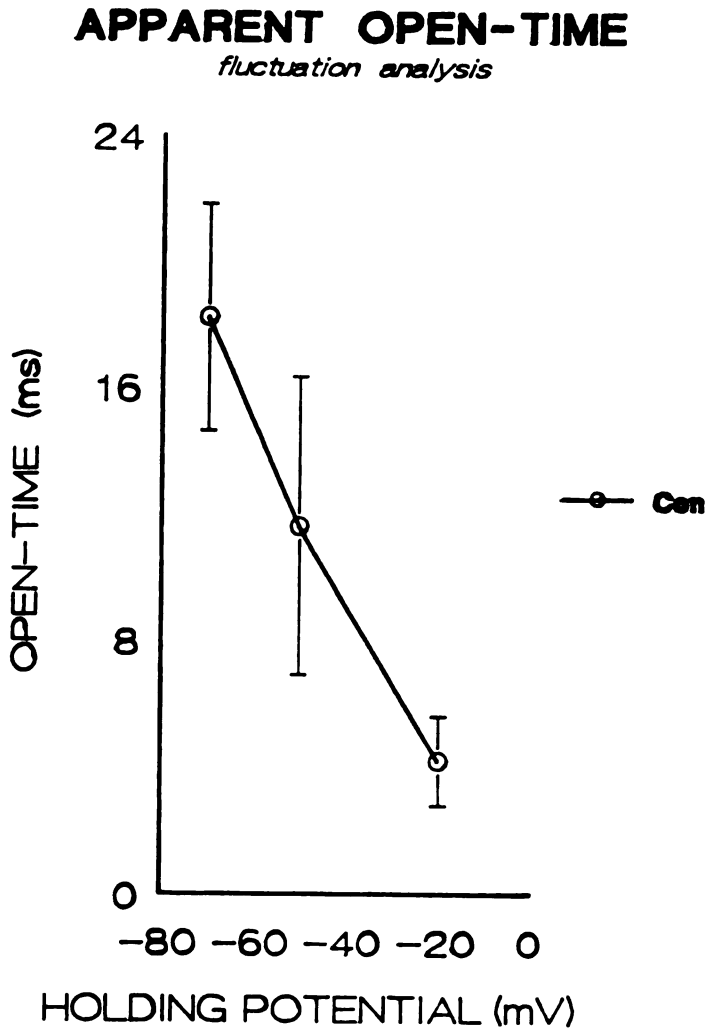


Figure 43. Values of τ from control muscles.

Values of τ calculated from hemidiaphragms of control rats. ACh induced fluctuations in end-plate current were recorded at holding potentials from -20 mV to -70 mV and spectra were generated as described in Figure 42. The corner frequency (f_c) was determined from a single lorentzian curve fit to each spectra and τ calculated from $\tau = 1 / 2\pi f_c$.

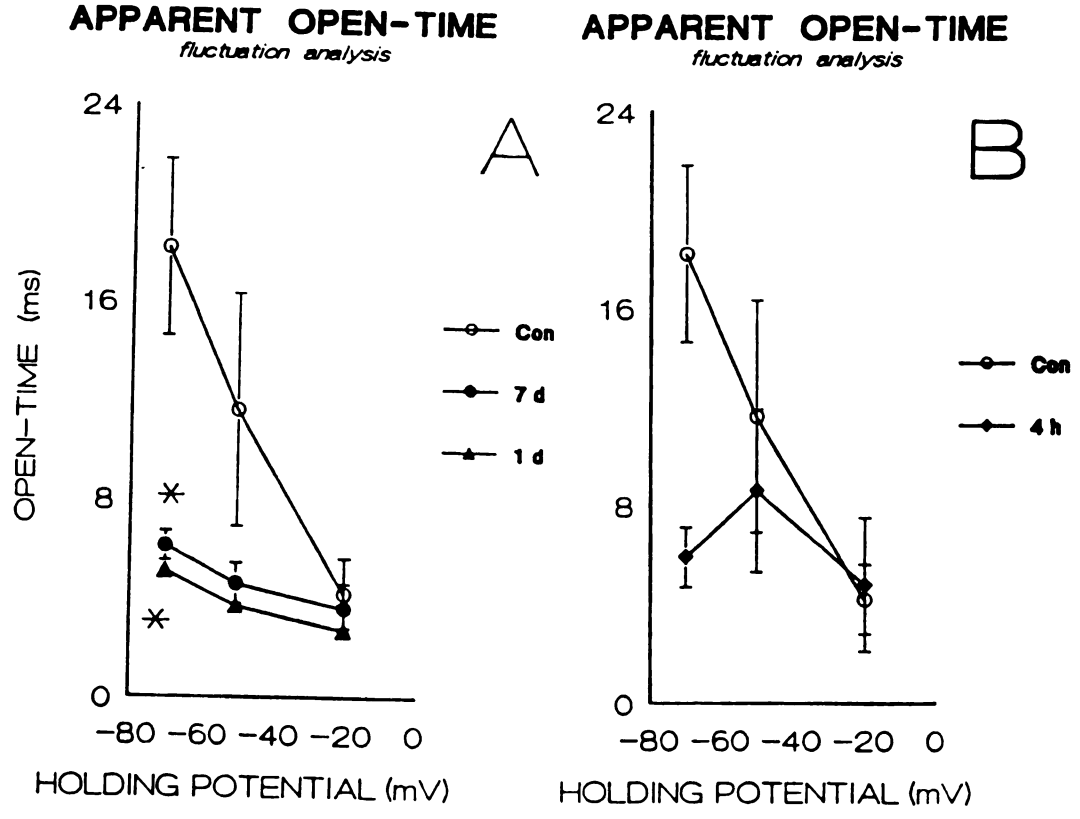


Figure 44. Effect of DTB on τ.

Effects of DTB on channel open-time (τ). ACh induced current fluctuations were recorded at membrane potentials between -20 and -70 mV in hemidiaphragm preparations taken (A) from rats treated 24 hours previously with a single 1 mg/kg dose of DTB and from rats treated chronically with DTB or (B) control rats and exposed to 1 μM DTB in the bathing medium. Values of τ were determined as in Figure 43.

Discussion.

By combining two microelectrode voltage clamp recording techniques with prolonged applications of ACh, via iontophoresis, we were able to record changes in the level of membrane noise during the application of ACh to end-plate regions. Spectra generated from the fluctuations in membrane noise, of control preparations, were similar to those observed by other groups examining ACh induced noise (Anderson and Stevens, 1973; Adams et al., 1981; Cull-Candy et al., 1988). Values of τ reported by Cull-Candy (1988) of 11 $\pm$ 0.9 at -80 mV are similar to values obtained in these studies. When compared to values of τ determined from single channel studies described earlier, these values appear high. It is questionable whether closures which occur within a burst of channel activity would be detected. Values of τ obtained from fluctuation analysis are believed to represent the duration of bursts for channels (Cull-Candy et al., 1988). τ values which were determined in the previous single channel studies (which described apparent open-time for individual openings within bursts) would be expected to be shorter than those determined in this study.

When the effects of DTB on channel behavior were examined it was found that the decay time constant τ calculated from muscles taken from rats exhibiting muscle weakness following chronic treatment with DTB (1 mg/kg/day) were decreased compared to τ values from control muscles. Similar effects on τ were observed in muscles taken from rats treated 24 hour previously with a single dose of DTB (1 mg/kg). When 1 μM DTB is applied in the bathing medium to muscles

from control rats τ also appears to be affected (at hyperpolarized holding potentials).

Conclusion. These results indicate that bursts of AChR-channel activity observed following iontophoresis of ACh onto muscles of rats treated with DTB are shorter than those observed in muscles of control rats. This is very similar to what was observed in the previous patch voltage clamp studies and could easily be explained by a similar mechanism. These effects are observed within 1 day following treatment (1 mg/kg of DTB) or as early as 4 hour following bath exposure to DTB (1 μM) as well as in muscles from rats treated chronically with DTB. When effects are monitored at the level of the single channel, changes in open-time are observed as early as 30 minutes after exposure to DTB (1 μM) and possibly as early as 5 minutes following exposure. The early times at which these effects are observed indicate that early changes begin to occur soon after exposure to DTB is initiated, and that these alterations may in part be responsible for the weakness observed with continued exposure.

Chapter 9. Discussion.

The initial observation that the rise and decay times for MEPPs were prolonged following exposure to DTB indicated that DTB may alter postsynaptic function. This effect was first observed in muscles taken from rats within 24 hour of a single 25 mg/kg dose. It was subsequently demonstrated that a similar prolongation of rise and decay times could also be detected in muscles taken from rats exhibiting muscle weakness following chronic treatment with DTB. Atchison (1989) reported that rise and decay times for EPPs were prolonged as well as for MEPPs. DTB could act in a variety of ways to cause the observed prolongation of synaptic potentials. Several potential modes of action for DTB will be examined in more detail in the following sections.

Cholinesterase inhibition. Following its initial release and binding to receptors further action of ACh is prevented by the presence of the enzyme AChE. This enzyme, which has an extremely high turnover rate for ACh, is also present in very high concentrations within the synaptic cleft (Cooper et al., 1982). This combination leads to the removal of presynaptically released ACh from the synaptic cleft within a few hundred microseconds. In most cases transmitter is released from the nerve terminal, diffuses across the synaptic cleft, binds a single time to receptors on the postsynaptic membrane leading to channel opening and then upon unbinding from the receptor is broken down very rapidly by AChE. It has been observed in several preparations that inhibition of AChE, by a variety of inhibitors, leads to an

increase in the amplitude and a prolongation of rise and decay times of synaptic potentials (Eccles and MacFarlane, 1948; Katz and Miledi, 1975; Laskowski and Dettbarn, 1979). Several lines of evidence go against DTB acting to prolong synaptic potentials via an inhibition of AChE. Nonspecific cholinesterase levels, both in plasma (Atchison and Peterson, 1981) and brain (Astwood et al., 1945), were found to be unchanged in rats exhibiting muscle weakness following chronic treatment with DTB. Following AChE inhibition there is an increase in MEPP and EPP amplitude, however, in muscles taken from rats following treatment with DTB EPP amplitude is decreased, while MEPP amplitude is either unchanged or deceased (Weiler et al., 1986; Atchison, 1989; Spitsbergen and Atchison, 1990). Cholinesterase inhibition also leads to pathological alterations in skeletal muscles which can be observed as early as 30 minutes following AChE exposure and which progress to encompass up to 7% of muscles in the body with chronic drug administration. Following 30 minutes of exposure to paraoxon (an irreversible cholinesterase inhibitor) mitochondria located within end-plate regions of muscle become dilated, sarcoplasmic reticulum is increased, subsynaptic folds become widened and fused and there is an increase in coated cleft vesicles. 24 hr following exposure to paraoxon, muscle fiber architecture displays a generalized disruption and there is an accompanying infiltration of phagocytes (Wecker et al., 1978). In contrast no evidence for pathological alterations are observed in muscles of rats exhibiting muscle weakness following chronic treatment with DTB (Astwood et al., 1945; Atchison et al., 1981b; Williams et al., 1987). It is interesting that at early

times following exposure of control muscles to a relatively high concentration of DTB (1.85 mM) in the bathing medium EPP amplitude, MEPP amplitude and MEPP frequency are increased and rise and decay times for MEPPs are prolonged. With continued exposure, EPP amplitude becomes decreased and block of evoked release is observed after approximately 30 minutes of exposure to DTB. At the time of block of transmitter release, MEPP frequency is still elevated from control levels (Spitsbergen and Atchison, 1990). An almost identical progression of events is observed following exposure of neuromuscular preparations to the cholinesterase inhibitor paraoxon. Within 30 minutes following paraoxon exposure, evoked release of transmitter is blocked, while MEPP frequency is increased and rise and decay times for MEPPs are prolonged (Laskowski and Dettbarn, 1979). AChE from Torpedo electroplax has been shown to contain a single free sulfhydryl group on its catalytic subunit which can react with a number of sulfhydryl reagents. Modification of this single sulfhydryl group by thiol reagents has been found to inactivate the enzyme (Steinberg et al., 1990). It is possible that a component of the acute effects observed following bath application of high concentrations of DTB is due to alterations in the activity of AChE. It appears unlikely that these effects are related to the effects observed in chronically-treated rats.

Alterations in synaptic morphology. At normal end-plates the structure of the synapses is very well defined. Presynaptic nerve terminals contain large numbers of transmitter-filled synaptic vesicles which are concentrated at specialized release sites known as active zones. These presynaptic active zones are located directly across

from postsynaptic specializations which contain large numbers of AChR molecules. In regions of synaptic contact pre- and postsynaptic specialized regions are separated by a very short distance, which allows transmitter released from the terminal to diffuse rapidly to postsynaptic receptors and initiate a depolarization of the end-plate region. Several studies which have examined the fine structure of neuromuscular junctions during the onset of muscle weakness in rats treated with DTB have reported alterations in terminal and synapse morphology following exposure to DTB. Kemplay (1984) demonstrated that retraction of the terminal arborization which led to a clumped appearance for nerve terminals was observed within 3 days of treatment of rats with DTB. Also observed at this time was an increase in terminal sprouting. Following 4 days of treatment with DTB terminal retraction and interposition of Schwann cell filopodia were observed (Jones, 1989). During terminal retraction or sprouting ACh could be released from regions which were no longer directly apposed to postsynaptic receptor sites. During retraction or sprouting the distance which ACh would have to diffuse prior to binding to receptors would be increased. It is possible that alterations in synapse morphology could be responsible for the observed prolongation of synaptic potentials observed following exposure to DTB. Effects on rise and decay times for MEPPs have been observed within 24 hour following a single dose of DTB (Spitsbergen and Atchison, 1990), a time at which no alterations in the structure of terminals or synapses are observed. Recent voltage clamp studies indicate that EPCs and the majority of MEPCs decay more rapidly in muscles from treated rats, an effect which would be unexpected if terminal retraction had

occurred. It is unlikely that the effects of DTB on postsynaptic function, which are responsible for the more rapid decay of synaptic currents, can account for the abnormally large, slow MEPCs which are observed in treated preparations. Instead it is likely that these aberrant MEPCs result from abnormal release of transmitter from retracting or sprouting terminals or from sites on normal terminals which do not normally release transmitter.

Effects on AChR-channels. Alterations in MEPP amplitude (Weiler et al., 1986; Spitsbergen and Atchison, 1990) and rise and decay times for synaptic potentials (Atchison, 1989; Spitsbergen and Atchison, 1990) were both indicative of possible alterations in postsynaptic function following exposure to DTB. It is known that the AChR contains disulfide groups which are critical to its function (Karlin and Bartels, 1968; Rang and Ritter, 1971). Finally, previous studies indicated that alterations in thiol/disulfide status within cells may be related to toxic effects of DTB (Williams et al., 1986). With these facts in mind, the studies described in previous chapters of this manuscript were undertaken. Results of these studies indicate that DTB does affect postsynaptic function.

Significance of effects on channel open-time. The results described in the previous sections of this manuscript indicate that exposure of AChR-channels to DTB alters these channels to decrease the time which they remain open. These alterations of decay kinetics appear to have little direct effect on transmission since the observed changes are similar following 1 day of treatment, when transmission does not appear to be affected and 7 days of treatment, when transmission is markedly affected. One

conclusion which could be drawn from these findings is that the decrease in single channel open-time has no physiological significance. This effect is observed in rats exhibiting weakness as well as those displaying no signs of weakness so it need not be related to the weakened state. Conversely, it could be concluded that these effects are some of the earliest changes observed following exposure to DTB and that by continuously altering these processes changes occur which lead to the observed weakness. The remainder of this discussion will be concerned with examining possible mechanisms by which subtle changes such as those observed could potentially lead to the observed weakness.

Trophic relationship between nerve and muscle. Traditionally the communication between muscle and nerve is thought to consist of transmitter release from the nerve terminal leading to depolarization of muscle end-plate region and muscle contraction. It is becoming more and more obvious that there is a fair amount of "cross-talk" between muscle and nerve. For instance, following block of transmission between nerve and muscle changes occur both at the level of the nerve and muscle, in an attempt to reestablish transmission. At the level of the muscle there is an increase in the number and distribution of AChR within the muscle membrane (Axelsson and Thesleff, 1959; Neher and Sakmann, 1976). At the level of the nerve terminal, sprouts are formed which can spread out from the original terminal and form new synapses on the same muscle fiber or surrounding fibers (Ironton et al., 1979; Kemplay, 1984). Following reinnervation, muscle fibers often become polyinnervated during the early stages of this process. Once transmission is

reestablished all multiply-innervated muscle fibers will become singly-innervated (Gorio et al., 1983). Recent evidence concerning the peptide transmitter calcitonin gene-related peptide (CGRP) indicates that this transmitter/modulator may act as a trophic message at the neuromuscular junction. CGRP is found in motor nerve terminals stored in dense-core vesicles (these dense core vesicles are increased in number in DTB treated rats (Jones, 1989)) which appear to be released under different stimuli from the small ACh filled vesicles (Matteoli et al., 1988). Effects of CGRP on neuromuscular transmission include a decrease in EPP and MEPP amplitude, quantal content of EPPs and sensitivity of the postsynaptic membrane to iontophoresed ACh (Caratsch and Eusebi, 1990), and an increase in the spontaneous release of MEPPs (Jinnai et al., 1989). CGRP has been reported to prevent disuse-induced terminal sprouting (Tsujimoto and Kuno, 1988). Thus, CGRP appears to play a role in the modulation of both pre- and postsynaptic function. Several studies have demonstrated that neuromuscular junctions which are regenerating, degenerating or physiologically-compromised exhibit an increase in very large slow MEPPs or MEPCs compared to normal junctions (see discussion of chapter on acute effects of DTB). It has been proposed that these abnormal MEPPs serve as a trophic signal between nerve and muscle. Evidence for this comes from studies examining neuromuscular junctions following botulinum toxin poisoning. In botulinum toxin-treated preparations the abnormal large MEPPs are the predominant form of transmitter released. It has been observed that even though neuromuscular transmission is completely blocked with this poisoning, the nerve and muscle

maintain trophic communication. If these preparations are treated with curare or pancuronium the trophic interactions are blocked indicating that ACh binding at nicotinic receptors is responsible for this communication (Mathers and Thesleff, 1978).

It is possible that the early subtle changes in channel behavior following exposure to DTB, which do not appear to alter transmission to a very large extent, could interact in some way with trophic communication between nerve and muscle. By continuously altering some signal between nerve and muscle, DTB could cause compensatory changes which then lead to the alterations in transmitter release observed in rats exhibiting muscle weakness. Another interesting mode of modulation has been proposed by Labarca et al. (1985), in which the nonquantal release of transmitter at neuromuscular junctions (Katz and Miledi, 1977) may regulate the responsiveness of the AChRs to the evoked release of transmitter. Here again the subtle changes which occur following exposure to DTB could over time cause compensatory changes which lead to the observed weakness.

Presynaptic nicotinic acetylcholine receptors. In early studies in which the effects of curare on transmission were examined, changes in transmission were observed which appeared to represent presynaptic effects of curare on transmitter release. These early studies indicated that ACh appeared to act presynaptically to mobilize transmitter stores (feed-forward mechanism) and that curare blocked this effect causing a decline in transmitter release during repetitive stimulation (Hubbard and Wilson, 1973; Glavinovic, 1979). Recently more evidence has been provided

that both nicotinic and muscarinic receptors are present presynaptically at the neuromuscular junction and that these receptors play a role in the modulation of transmitter release (Wessler, 1989; Bowman, et al., 1990). There is still debate whether these receptors, if present, exert positive or negative feedback control over transmitter release (Storella and Bierkamper, 1990).

If AChRs are present on the nerve terminal and if binding of ACh to these receptors modifies transmitter mobilization or release, then this could represent a target for DTB at which the subtle changes at postsynaptic receptors could have a far greater impact on transmitter release.

DTB-induced muscle weakness as a model of human neuromuscular disorders. The neuromuscular weakness observed in rats treated chronically with DTB is accompanied by changes in transmission which are similar in many ways to those observed in human patients suffering from a variety of neuromuscular disorders. Similarities which exist include a decrease in *m* for nerve evoked EPPs and EPCs, which is also observed in Lambert Eaton Myasthenic Syndrome, Congenital AChE deficiency, Botulism and several other disorders related to myasthenia gravis (Engle et al., 1977; 1990). A decrease in MEPP amplitude which is observed in muscles from treated rats and is also observed in Congenital AChE deficiency, slow channel Myasthenic Syndrome and Congenital AChR deficiency. A slowing of the MEPP decay is observed in Slow-channel Myasthenic Syndrome, while more rapid decay of synaptic currents is observed in High-conductance fast-

channel Syndrome and AChR deficiency and short channel open-time (Engel et al., 1982; 1990). Although the alterations in transmission observed in rats exhibiting weakness following treatment with DTB do not exactly mimic all of the deficits observed in any one of the aforementioned syndromes, they do provide many similarities to a number of these states. A common aspect of most of these syndromes is the reduction in the safety factor for transmission in patients afflicted with these diseases. Patients suffering from these syndromes appear normal when at rest, however, during even moderate activity they exhibit increasing degrees of skeletal muscle weakness. A similar reduction in safety factor has been observed in muscles from rats treated with DTB. Atchison (1990) demonstrated that a reduction in safety factor and abnormal quantal secretion could be detected prior to muscle weakness in neuromuscular preparations from rats treated with DTB. By understanding how DTB interacts with different components of the nerve and muscle and how these interactions lead to the weakness observed in treated rats a better understanding of the causes of the weakness in the human disorders may be gained.

Conclusion

The results of the experiments described above indicate that exposure to DTB alters the time which nicotinic AChR-channels remain open. Changes in channel open-time are some of the earliest signs observed during treatment of rats with DTB. It is possible that the changes observed if maintained by continued exposure to DTB

could lead to the alterations responsible for the weakness observed in chronically treated rats. Perhaps by altering trophic communication between nerve and muscle or by altering sensitivity of presynaptic nicotinic receptors which modulate release of ACh.

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