An Analysis of the Sites of Action of Theophylline at the Neuromuscular Junction of the Cat

Patricia L. Shinnick
Loyola University Chicago

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AN ANALYSIS OF THE SITES OF ACTION OF THEOPHYLLINE AT THE NEUROMUSCULAR JUNCTION OF THE CAT

by

Patricia L. Shinnick

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

February

1974

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ACKNOWLEDGMENTS

I would like to express my sincerest appreciation to Dr. R.S. Jacobs for his guidance throughout my graduate training. I am also grateful to Dr. J. Gallagher and Dr. S. Nishi for their interest and assistance in this project.

I am indebted to Dr. A.G. Karczmar, Professor and Chairman of the Department of Pharmacology, for the opportunity to study at Loyola and for his interest and encouragement in my graduate training. I would also like to thank Mrs. Geraldine Messina, Dr. K. Kuba, Dr. S. Minota, Dr. N. Tashiro, and Dr. H. Higashi for their interest and help.

I would like to extend my deepest gratitude to my family for their continuous support and encouragement in this endeavor.
BIOGRAPHY

The author, Patricia L. Shinnick, is the daughter of Richard Charles Shinnick and Dorothy (Malkewicz) Shinnick of Peru, Illinois. She was born on July 28, 1947 in Chicago, Illinois.

Ms. Shinnick obtained her secondary education at LaSalle-Peru Twp. High School, Peru, Illinois where she graduated in June, 1965.

After completing the year of pre-pharmacy at Milliken University, Decatur, Illinois, she entered the University of Illinois College of Pharmacy in September, 1966. While at the University of Illinois she was elected to Rho Chi pharmacy honor society and Phi Kappa Phi all university honor society. In June, 1970, she graduated, cum laude, with a Bachelor of Science degree in Pharmacy.

In July, 1970, she passed the state licensure examination and is a Registered Pharmacist in the State of Illinois.

In September, 1970, Ms. Shinnick was accepted in the Department of Pharmacology at the Stritch School of Medicine, Loyola University and placed under the direction of Dr. R.S. Jacobs. The following is a list of publications which have appeared in the literature as a result of her studies at Loyola:

Pharmacologist, 13, 264.


Ms. Shinnick is looking foreward to continuing her education under the direction of Dr. S. Nishi in his neurophysiology laboratories here at Loyola.
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<td>Å</td>
<td>angstrom</td>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>Ca</td>
<td>calcium; Ca$^{++}$; calcium ion</td>
</tr>
<tr>
<td>ChE</td>
<td>cholinesterase</td>
</tr>
<tr>
<td>Cl</td>
<td>chloride; Cl$^{-}$; chloride ion</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>cyclic AMP</td>
<td>adenosine 3', 5' monophosphate</td>
</tr>
<tr>
<td>d$_m$</td>
<td>mobilization rate</td>
</tr>
<tr>
<td>dTc</td>
<td>d-tubocurarine</td>
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<tr>
<td>EPP</td>
<td>equilibrium potential of the endplate potential</td>
</tr>
<tr>
<td>EPP</td>
<td>endplate potential</td>
</tr>
<tr>
<td>G$_m$</td>
<td>membrane conductance</td>
</tr>
<tr>
<td>g$_K$</td>
<td>potassium conductance</td>
</tr>
<tr>
<td>g$_{Na}$</td>
<td>sodium conductance</td>
</tr>
<tr>
<td>head</td>
<td>first fifteen EPPs in a train</td>
</tr>
<tr>
<td>K</td>
<td>potassium; K$^+$; potassium ion</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>M$_0$</td>
<td>quantal content of the first EPP in a train</td>
</tr>
<tr>
<td>MEPP</td>
<td>miniature endplate potential</td>
</tr>
<tr>
<td>Mg</td>
<td>magnesium; Mg$^{++}$; magnesium ion</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>min</td>
<td>minute</td>
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<td>ml</td>
<td>milliliter</td>
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<td>mm</td>
<td>millimeter</td>
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<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>msec</td>
<td>millisecond</td>
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<tr>
<td>mV</td>
<td>millivolt</td>
</tr>
<tr>
<td>MΩ</td>
<td>megohm</td>
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<tr>
<td>Na</td>
<td>sodium; Na⁺; sodium ion</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>p</td>
<td>probability of release</td>
</tr>
<tr>
<td>q</td>
<td>quantum size</td>
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<tr>
<td>R</td>
<td>membrane resistance</td>
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<tr>
<td>R_m</td>
<td>resistance for a unit area of membrane</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>tail</td>
<td>last EPPS in a train excluding the head</td>
</tr>
<tr>
<td>T_m</td>
<td>membrane time constant</td>
</tr>
<tr>
<td>μ</td>
<td>microns</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>V_K</td>
<td>potassium equilibrium potential</td>
</tr>
<tr>
<td>V_Na</td>
<td>sodium equilibrium potential</td>
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<tr>
<td>Ω</td>
<td>ohm</td>
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CHAPTER I

INTRODUCTION
CHAPTER I

INTRODUCTION

In prior investigations in our laboratory, theophylline was shown to reverse muscle fatigue in biopsy samples obtained from patients diseased with myasthenia gravis. Based on that finding, the sites and possible mechanisms of action of theophylline were investigated at the neuromuscular junction of cat tenuissimus muscle using intracellular microelectrode techniques.

Experiments were initially carried out to study the effect of theophylline on spontaneously released miniature endplate potentials (MEPPS). Theophylline increased MEPP amplitude, time course, and frequency. A change in MEPP frequency indicates a presynaptic effect while alterations in MEPP amplitude and/or time course indicate either a post- and/or presynaptic site of action.

To study the effect of theophylline on evoked release, muscle contraction was blocked by treatment with d-tubocurarine (dTc) or by transversally cutting the muscle fibers. The evoked endplate potential (EPP) amplitude and time course increased in both preparations after treatment with theophylline.

Trains of EPPS were employed to investigate the effect of theophylline on presynaptic release. In dTc-immobilized preparations,
presynaptic facilitation of evoked release was not observed. A presynaptic facilitatory effect was unmasked, however, in the cut muscle fiber preparation as evidenced by an increased probability of release and decreased readily available stores.

A postsynaptic site of action of theophylline was identified, since the response to exogenously applied acetylcholine (ACh) was potentiated. Carbachol responses were also potentiated but to a lesser degree than the ACh responses, suggesting that some of the increase in MEPP and EPP amplitude seen with theophylline treatment may be due to cholinesterase (ChE) inhibition. Inhibition of ChE may also be responsible for the theophylline-induced prolongation of the MEPP and EPP time course.

Passive membrane characteristics were also investigated since not all of the postsynaptic effects of theophylline could be attributed to ChE inhibition. In these studies membrane resistance in normal and cut muscle fiber preparations was found to decrease with theophylline treatment. This decreased resistance with the concurrent increase in ionic permeability of the membrane, may be responsible for the membrane depolarization which occurred in all experiments with theophylline treatment.

Subsynaptic characteristics were also examined since a movement of the equilibrium potential of the EPP ($E_{EPP}$) to a more positive level would increase MEPP and EPP amplitude. It was found that
theophylline produced such a positive shift in the $E_{EPP}$ coupled with ChE inhibition may account for the increase in amplitude and prolongation of the time course of the MEPP and EPP.

Aside from the presumed ChE inhibition, the pre- and post-synaptic actions of theophylline appear to possess a common link; i.e., in all cases, the effects of theophylline seem to resemble those effects which would be produced if cellular calcium had been mobilized.
CHAPTER I. INTRODUCTION

A. ANATOMY

The cat most closely resembles man in its response to drugs at the neuromuscular junction (Bowman, 1962). The tenuissimus muscle in the cat described as "a most beautiful muscle in the hind limb" by Brown (1913), is ideal for intracellular microelectrode studies due to its slender nature and distinct nerve supply.

Tenuissimus Nerve-Muscle Preparation

In the cat, the tenuissimus muscle (abductor cruris caudalis) is located in the ventral hind limb. It has its origin on the tip of the transverse process of the second caudal vertebrae and passes obliquely beneath the biceps femoris to fuse with the ventral border of the biceps where they insert in the same fascia (Reighard and Jennings, 1935); (see Figure 1). This long, slender, "ribbon-like" muscle varies from 9-15 cm in length and 2-4 mm in width; the single fibers are arranged in an interdigitating fashion and are approximately 2 cm in length (Adrian, 1925). According to Porter and Hart (1923), a cross-section of muscle contains 1000 fibers. Boyd (1956) determined that the mean diameter of the fibers in cross section is 30μ with a mean surface fiber diameter of 35μ. By correcting for shrinkage, Boyd and Martin (1959)
Figure 1. Lateral view of the tenuissimus muscle (abductor cruris caudalis) in relation to the sciatic nerve (ischio-taical). (With permission of the author and publisher, A Study of the Cat with reference to man, Figure 4-13).
estimated that the diameter of the surface cells was 50μ.

The nerve to the tenuissimus emerges from the sciatic and bifurcates into two branches before entering the muscle 2 to 3 cm from the origin. Adrian (1925) observed that the individual fibers in the main nerve sent branches to both ends of the muscle. Cooper (1929) further concluded that a single nerve fiber supplies a chain of muscle fibers enabling simultaneous contraction. This pattern of innervation, in the cat, permits the tenuissimus to act as a fast white twitch muscle (Maclagan, 1962) which functions in the flexor reflex (Sherrington, 1910) where it aids in the abduction of the thigh and flexion of the shank.

The Neuromuscular Junction

The neuromuscular junction, including the area of the motor nerve terminal, the endplate region of the muscle, and the intervening cleft has been termed "synapse" (Sherrington, 1897). Although the synapse of the cat tenuissimus muscle has never been studied with electron microscopy, it is said to conform to the descriptions given for other mammalian species (Boyd, 1956).

Couteaux (1958) described the basic structure of the neuromuscular junction. He found that the terminal part of the motor axon which had lost its myelin sheath was still covered with Schwann cells as it occupied the synaptic troughs. These troughs joining the axoplasm and sarcoplasm contained a palisade subneural apparatus.
With the introduction of the electron microscope, distinct pre- and postsynaptic membranes were described (Palade, 1954; Robertson, 1954, 1956; Reger, 1955). It was found that the axoplasmic and sarcoplasmic membranes were separated by a synaptic cleft (450\text{-}600\text{Å}) (Robertson, 1956; Reger, 1957; Andersson-Cedergren, 1959). In the motor nerve terminal "vesicles" were first observed by Palade (1954). The majority of vesicles have a membrane 40\text{-}50\text{Å} thick and mean diameter of 500\text{Å} (Hubbard, 1970). Synaptic vesicles were observed to have the highest concentration close to the membrane opposite the synaptic cleft (Hubbard and Kwanbunbumpen, 1968; deHarven and Coers, 1959; Birks, Huxley, and Katz, 1960). The post-junctional folds were first described by Palade (1954) and Robertson (1954) and seen to constitute the subneural lamellae of Couteaux (1958). Histochemical studies coupled with electron microscopy have shown that acetylcholinesterase (AChE) is located chiefly on the external surface of this structure (Barnett, 1962; Salpeter, 1967). At the neuromuscular junction, AChE has also been shown to be present on the prejunctional axoplasmic membrane (Davis and Koelle, 1967) and associated with the vesicular structures in the presynaptic nerve terminals (Zacks and Blumberg, 1961; Miledi, 1964). Barnett (1962) also found evidence of AChE staining presynaptically, however, he suggested that the presence of AChE at these locations was due to fixation artifact.

Recently, synaptic areas in living frog muscle were viewed
using Nomarski differential interference contrast optics (McMahan, Spitzer, and Peper, 1972). The myelinated axons were seen to give rise to unmyelinated terminal branches 1-3μ in diameter, which extend longitudinally along the muscle fiber for 300μ. The nerve terminal could be removed using collagenase revealing the subsynaptic membrane, a living representative of Couteaux's (1958) work. Figure 2 is a photomicrograph of the tenuissimus muscle as viewed under Nomarski optics.
Figure 2. Photomicrograph of kitten tenuissimus muscle under 400X magnification using Nomarski optics. NT = nerve terminal.
CHAPTER I. INTRODUCTION

B. PHYSIOLOGY

The function of the neuromuscular organization is to transmit impulses from the motor nerve to the muscle fiber. This communication system is dependent on the properties of the nerve, muscle, and synapse.

Nerve and muscle fibers are cylindrical conductors surrounded by membranes which endow them with properties similar to that of a cable (Hermann, 1899). They possess the characteristics of resistance, and capacitance common to a cable. If a current is passed through a fiber, a voltage change, i.e., an electrotonic potential is induced in the fiber. The voltage does not immediately change but lags behind the current with a time constant dependent on the resistance and capacitance of the membrane (Katz, 1966).

These cable conductors confined by selectively permeable membranes determine the effectiveness of the transmission process.

The Membrane Concept

The basic concept on which electrophysiology is founded is that cells have an electrochemical barrier, the membrane, limiting the
diffusion of molecules and charged moieties (Hubbard, Llinas, and Quastel, 1969). The major ionic constituents on the outside of nerve and muscle cells are Na and Cl; inside the cell these ions amount to less than 15% of the electrolytes. Intracellular K is accumulated 20 to 50 times higher than that of the external media, and impermeable anions constitute the rest of the intracellular ionic environment (Katz, 1966). Bernstein (1902) proposed that the origin of the resting membrane potential was due to a selective permeability to potassium ion and that this selectivity was lost during the action potential resulting in a neutralization of the membrane potential.

**Resting Membrane Potential**

Bernstein's hypothesis is supported by the observations that in altered potassium concentration, the resting membrane potential was found to change in the predicted manner (Curtis and Cole, 1942; Adrian, 1956). In opposition to his theory, Boyle and Conway (1941) showed that the muscle membrane was permeable to K and Cl, and that their distribution across the membrane followed a Donnan equilibrium. In contrast, Cl ion is not distributed according to a Donnan equilibrium in motor nerve fibers due to the presence of a Cl pump (Keynes, 1963). Tracer experiments also contradicted the concept of selective permeability since Na also appeared to cross the nerve and muscle membrane (Ussing, 1947; Hodgkin and Horowicz, 1959). In 1955, Hodgkin and
Keynes showed that the efflux of sodium in nerve was due to an active process by means of which Na ions are extruded from the cell. Similarly, single muscle fibers were found to be permeable to Na, as well as K and Cl (Hodgkin and Horowicz, 1959).

In 1943, Goldman formulated an equation encompassing the permeability of Na, K and Cl ions in the determination of membrane potential which remains unchallenged today. In most mammalian species, the potential generated across the muscle membrane by the selective permeability of ions ranges from -60 mV to -90 mV; the mean resting membrane potential for cat tenuissimus muscle is -75 mV (Boyd and Martin, 1956a).

The Action Potential

The ionic mechanism of the action potential as proposed by Bernstein (1902) was thrown into doubt when Hodgkin and Huxley (1939) showed that the excitation leads to a transient reversal (i.e. an overshoot), not simply a neutralization of the resting potential. This led to an extensive re-investigation of the mechanism underly­ing the action potential by many investigators.

Initiation of the action potential requires a local or decremental depolarization from a stimulus to bring the resting membrane potential to a critical level, i.e. an ignition point or threshold potential (Katz, 1966). At this point the response becomes an "all or nothing" phenomenon; the membrane develops a transient specific permeability
to Na ions (Hodgkins, 1958). As a consequence of the permeability change, the Na ions move inward along an electrochemical gradient determined by the equilibrium level for Na, $V_{Na}$ (Hodgkin and Huxley, 1952a). $V_{Na}$, however, is not reached, as the membrane develops a delayed increase in K conductance (Hodgkin and Huxley, 1952b). This leads to a rapid reversal of the action potential to the initial level, inactivating the Na conductance. The raised K conductance and inactivation of the Na carrying system persist for a few milliseconds after the spike giving rise to the refractory period (Hodgkin, 1958). In this state, the membrane potential is driven toward $V_K$. In the case of nerve fibers, in which $V_K$ is below (more negative than) the resting membrane potential, a "positive" after hyperpolarization results (Hodgkin, 1958); in muscle, $V_K$ is equal to the resting membrane potential (Katz, 1966).

At the neuromuscular junction, each nerve action potential is followed by a similar impulse in the muscle. The current generated by the action potential in the nerve terminal is followed 0.5 to 0.8 msec later by another current which arises locally in the postsynaptic membrane (Katz and Miledi, 1965a). The presence of this synaptic delay indicates not only an anatomical but also an electrical discontinuity between nerve and muscle. Evidently, the electrical process comes to a halt at the motor nerve terminal and another non-
electrical process intervenes before electrical conduction resumes in the muscle. The coupler between the pre- and postsynaptic events has been shown by many investigators to be cholinergic chemical transmission.

**Cholinergic Chemical Transmission**

The first evidence for cholinergic chemical transmission resulted from the work of Hunt (1901) who suggested that a blood pressure lowering substance found in adrenal glands was an ester of choline. Subsequent studies of Hunt and Taveau (1906) proved that an acetate ester of choline had 1000 x the depressor action of choline itself. Langley (1909) postulated from his work with curare and nicotine that motor nerve stimulation affects a receptor substance on muscle by releasing a chemical. In 1914, Dale concluded that the short duration of action of acetylcholine was due to its enzymatic destruction. The classic experiments of Loewi (1921) demonstrated for the first time the release of a chemical "Vagustoff" during nerve stimulation. The failure to identify "Vagustoff" as acetylcholine was due to the fact that acetylcholine was not as yet shown to be a constituent of the body. Dale and Dudley (1929) succeeded in isolating and characterizing acetylcholine in spleen extracts. Dale, Feldberg, and Vogt (1936) extended the work of Otto Loewi to demonstrate that acetylcholine is released upon stimulation of skeletal motor nerves.
Synthesis of acetylcholine (ACh) requires the presence of various factors including choline, coenzyme A and active acetate. Coenzyme A combines with acetate activated by chemical energy supplied in the form of adenosine triphosphate (Berg, 1956a, b). Choline concentration is sufficient to support optimal synthesis. A variety of investigators have shown that there is a specific transport system, probably located in the presynaptic membrane or in intracellular particles within the nerve endings that acts to carry extracellular choline to intracellular sites (Birks and MacIntosh, 1961; Hebb, Knjefvic, and Silver, 1964; Potter, 1968). A specific enzyme, choline acetyl transferase (ChAc) (Nachmansohn and Machado, 1943) is capable of transferring the acetyl groups from coenzyme A to choline, forming acetylcholine. There is evidence that ChAc and ACh are formed within the soma and are transported to the nerve endings by axoplasmic flow (Hebb and Waites, 1956; Hebb and Silver, 1963). ChAc appears to be in the soluble cytoplasmic fraction (Fonnum, 1968; Whittaker, 1968). Vesicles have been isolated and shown to contain ACh; ACh may therefore be synthesized in the cytoplasm and transferred to the vesicles (Whittaker, 1968).

Direct evidence for a vesicle hypothesis has been found at the neuromuscular junction of the rat diaphragm. The number of vesicles close to the presynaptic membrane is increased after prolonged low frequency stimulation (Jones and Kwanbunbumpen, 1968), but reduced
after prolonged high frequency stimulation (Hubbard and Kwanbunbumpen, 1968).

The compartmentalization of ACh in the nerve terminal was investigated by Birks and MacIntosh (1961). Based on their findings in the ganglion, they suggested that there was a pool of ACh located in the cytoplasm which is not released by nerve stimulation. Another depot, vesicular ACh, consisting of preformed transmitter, represented the ACh fraction which was present when synthesis was blocked with hemicholinium drugs. Vesicular ACh was found to be composed of two subfractions, one of which was smaller and more readily liberated than the other. A third intracellular store termed "surplus" ACh did not make an important contribution to release since it could only be found when ChE was inactivated.

At high rates of stimulation the amount of acetylcholine released falls to a level at which the output per minute is constant (Perry, 1953; Birks and MacIntosh, 1961). This phenomenon is reflected in trains of endplate potential (EPP amplitudes which show an initial rapid rundown at the beginning of stimulation followed by EPPS of lower sustained amplitude (Liley and North, 1953; Elmqvist and Quastel, 1965b). These observations led to the hypothesis that there exists in the nerve terminal a readily releasable store of ACh
which is replenished by a less mobile store.

In addition to finding release of ACh on nerve stimulation, the transmitter substance was also detected in the absence of nerve stimulation providing evidence for continuous or spontaneous release (Straughan, 1960; Knjevic and Mitchell, 1961).

Transmitter Release

Two types of release of transmitter are evident at the neuromuscular junction: 1) spontaneous release and 2) evoked release.

Spontaneous Release:

In the absence of nerve impulses there is spontaneous release of acetylcholine from the nerve terminals giving rise to minute voltage fluctuations in the region of the motor endplate (Fatt and Katz, 1952). These miniature endplate potentials (MEPPS) result from the local depolarizations of the motor endplate by amounts of ACh too small to elicit a propagated muscle action potential. MEPPS have been detected at skeletal muscle synapses in a variety of species, e.g., in the frog (Fatt and Katz, 1952; Burke, 1957), cat (Boyd and Martin, 1956a), rat (Liley, 1956a), fish (Takeuchi, 1959), human (Elmqvist, Johns, and Thesleff, 1960), and avian muscle fibers (Ginsborg, 1960).

These spontaneous potentials are characterized by two parameters:
frequency and amplitude.

MEPP amplitude is primarily a function of the properties of the subsynaptic membrane (Katz, 1962). At a particular synapse, the amplitude of MEPPS show little variation around their mean of 0.5 mV (Fatt and Katz, 1952; Boyd and Martin, 1956a; Liley, 1956a) with variations predicted by a Gaussian or normal distribution. There is a small incidence of MEPPS released termed "giant" MEPPS which are a multiple of the mean MEPP amplitude (Liley, 1957). Variability in the amplitude of MEPPS from synapse to synapse have been explained as variations in the postsynaptic properties of the fiber due to differences in cell diameter (Katz and Thesleff, 1957); i.e. the smaller the muscle fiber, the larger the size of the MEPPS recorded. Katz and Thesleff (1957) also found that these smaller fibers have a larger membrane resistance. Since the average quanta of ACh generates approximately the same amount of ionic current (Takeuchi and Takeuchi, 1960b), the voltage recorded (MEPP amplitude) becomes greater the higher the resistance of the muscle membrane. Postsynaptic alterations in MEPP amplitude at a single synapse may result from a change in the subsynaptic action of the transmitter: 1) inhibition of AChE, 2) change in receptor density, 3) change in the active membrane characteristics, i.e. the equilibrium potential of the endplate determined by specific ionic conductances, and 4) change in the passive membrane characteristics, i.e. the resistance.
Since MEPPS originate presynaptically, their frequency indicates only presynaptic phenomena (Katz, 1962). By sequentially recording MEPPS, Fatt and Katz (1952) observed that the occurrence of MEPPS was statistically predictable and followed a Poisson distribution, i.e. MEPPS occurred as a random series of events in which the probability of any one event occurring is constant and does not depend upon a prior event. The probability of \( x \) events occurring in a particular time interval is given by:

\[
P(x) = \frac{e^{-m} \cdot m^x}{x!}
\]

(Katz, 1969)

where \( e \) = natural logarithm base, \( m \) = mean number of events at a particular time, and \( x \) = number of events. The incidence of that number of events in a total population \( N \) is:

\[
n = N \cdot P(x)
\]

(Katz, 1969)

In a Poisson distribution, the variance is equal to the mean; therefore, the mean frequency, \( \bar{f} \) in \( t \) seconds is:

\[
\bar{f} = \frac{N \pm \sqrt{N}}{t}
\]

(Hubbard, Llinas and Quastel, 1969)

A presynaptic alteration in the frequency of MEPPS may be due to several mechanisms. Depolarization of the nerve terminal evokes an increase of MEPP frequency (del Castillo and Katz, 1954b; Liley, 1956c; Hubbard and Willis, 1962b); hyperpolarization, in contrast, produces either a decrease in frequency (Liley, 1956c; Hubbard and Willis, 1962a) or no change in frequency (Landau, 1968). Raising
osmotic pressure increases frequency, while hypotonicity decreases it (Fatt and Katz, 1952; Boyd and Martin, 1956a; Liley, 1956a).

Frequency is similarly affected by temperature changes (Fatt and Katz, 1952; Boyd and Martin, 1956a; Hofmann, Parsons, and Feigen, 1966), i.e. increased temperature increases frequency and decreased temperature lowers it.

**Evoked Release:**

The generation of an endplate potential is an intermediary between nerve and muscle action potentials. The initiating factor in evoked release is independent of the ionic fluxes of Na and K, since tetradotoxin which blocks the Na flux (Narahashi, Moore, and Scott, 1964) and tetraethylammonium (TEA) which blocks the K flux (Hagiwara and Saito, 1959) do not block the release by depolarizing pulses applied to nerve terminals (Katz and Miledi, 1965c, 1967b; Ozeki, Freeman, and Grundfest, 1966; Bloedel, Gage, Llinas, and Quastel, 1966; Kusano, Livengood, and Werman, 1967). The mechanism of release involves the influx of calcium into the presynaptic nerve terminal (Harvey and MacIntosh, 1940; Hodgkin and Keynes, 1957; Birks and MacIntosh, 1957). The endplate potential that follows nerve terminal stimulation has been shown to consist of summated quantum components (MEPPS). Evidence for this hypothesis was obtained when the endplate potential was fractionated into smaller
units by lowering Ca or raising Mg in the bathing media (del Castillo and Engbaek, 1954). The evoked EPPS were identical in amplitude to the MEPPS recorded simultaneously. Del Castillo and Katz (1954a) further showed that when EPPS were fractionated in this manner the amplitudes of the EPPS in a long series of evoked responses was (like MEPP frequency) predictable by the Poisson formula.

If, under normal ionic conditions, repetitive stimulation is applied, the amplitude of each successive EPP falls to a plateau size (Liley and North, 1953; Hubbard, 1963; Elmqvist and Quastel, 1965b). This early tetanic rundown is due to a progressive depletion of the immediately available store (N) by the repeated stimuli. The extent of the depression depends on the stimulus interval and on the number of quanta released by the preceding impulse and is thought to be a presynaptic phenomenon (Otsuka, Endo and Nonomura, 1962; Elmqvist and Quastel, 1965b; Thies, 1965). The quantum content (m) of the EPPS does not fall to zero because the store is replenished by refilling or mobilization processes.

Each nerve action potential releases only a fraction (p) of the releasable store of quanta (N) (Liley and North, 1953). The mean number of quanta (m) released by each action potential is predicted by the formula:

\[ m = N \cdot p \]  
(del Castillo and Katz, 1954a)
Thus, it seems that the nerve action potential coincident with the influx of calcium ion into the nerve terminal markedly increases the probability of release (p) so that several hundred quanta leave their presynaptic sites almost simultaneously.

**Function of Acetylcholine**

Unlike the electrically sensitive muscle membrane, the chemically sensitive endplate region is depolarized by the action of ACh released from the nerve terminal (Fatt and Katz, 1951). The interaction of acetylcholine with receptor molecules in the endplate region of the postsynaptic membrane produces a decrease in local membrane resistance accompanied by a flow of ionic current through the activated membrane (Fatt and Katz, 1951; del Castillo and Katz, 1954c). The potential change produced across the motor endplate is a decremental depolarization, not the self-propagating, all or none phenomena characteristic of an action potential. During the action of the transmitter the endplate becomes permeable to both Na and K ions simultaneously but not to Cl (Takeuchi and Takeuchi, 1960a). The membrane potential at which the EPP is nullified does not correspond to the equilibrium potential of any of the three principle ionic channels, Na, K, or Cl, since the reversal point for the EPP is -15 mV (del Castillo and Katz, 1954c). The equilibrium potential of the endplate is that membrane potential at which the sodium and potassium currents are
equal and opposite in direction, according to the following equation:

\[ E_{\text{EPC}} = \frac{V_K + \Delta g_{Na}/\Delta g_K \cdot V_{Na}}{1 + \Delta g_{Na}/\Delta g_K} \]  

(Takeuchi and Takeuchi, 1960a).

Identical values for the equilibrium potential have been obtained using the direct collision technique (del Castillo and Katz, 1954c), the constant current technique (Takeuchi and Takeuchi, 1959) and the constant voltage (Voltage clamp) technique (Takeuchi and Takeuchi, 1959).

Membrane Sensitivity to Acetylcholine

The effects of nerve stimulation can be mimicked by using an electrically controlled micropipette filled with ACh (Nastuk, 1953). When ACh is applied extracellularly to the surface of the endplate membrane, a depolarization (ACh potential) is produced (Nastuk, 1953; del Castillo and Katz, 1955). Del Castillo and Katz (1955) concluded that there were specific ACh receptors attached to the outer surface of the endplate membrane, while the remainder of the membrane was insensitive and impermeable to ACh.

In mammalian muscle, the area of membrane sensitive to iontophoretic application of ACh extends several hundred microns beyond the synapse; however, the greatest sensitivity to ACh occurs at
the subsynaptic region of the neuromuscular junction where MEPPS can be recorded. Sensitivity to ACh is several thousand times lower beyond the subsynaptic region (Miledi, 1960).

According to Feltz and Mallart (1971a), the junctional and extra-junctional responses constitute two groups of receptors. The junctional receptors show high sensitivity to ACh and the recorded potential exhibits a short rise-time that does not increase as the ACh dose is increased. On the other hand, extrajunctional receptors show a relatively low sensitivity to ACh and slow rise-time that increases with increases in ACh dose. Furthermore, non-focal recordings of ACh potentials result in a low equilibrium potential (~42 mV) (Feltz and Mallart, 1971b). Using Nomarski optics to precisely locate the nerve terminal's edge, the sensitivity of the endplate was found to decline 10% when ACh was applied 5-10μ from the terminal's edge; based on these data, it was estimated that only 3% of the ACh response was contributed by extrajunctional receptors (Peper and McMahan, 1972).

Role of Calcium

Presynaptic Phenomena:

Spontaneous release is not abolished in the absence of Ca; however, MEPPS are sensitive to increasing concentrations of Ca (Fatt and Katz, 1952; Boyd and Martin, 1956a; Hubbard, 1961; Hubbard, Jones, and Landau, 1968a). In rat diaphragm, 30% of the
MEPPS released in normal Ca Ringer solution are independent of the Ca concentration (Hubbard, Jones and Landau, 1968a). Hubbard, et al. (1968a, b) suggested that the rate of spontaneous release is accelerated due to the presence of Ca.

In contrast to spontaneous release, the evoked release of ACh is abolished in the absence of Ca (del Castillo and Stark, 1952; Liley, 1956b). Using focal application of Ca, Katz and Miledi (1967c) have shown that this ion must be available during the nerve action potential if release is to occur. This calcium dependent release is not associated with any change in the amplitude or duration of the nerve action potential (Katz and Miledi, 1965b). Moreover, the acceleration of MEPP frequency by high concentrations of K ions is dependent upon the presence of calcium and is inhibited by Mg (Hubbard, 1961). The fact that calcium does not change the extent of the membrane depolarization produced by potassium ions, indicates that the effect of Ca is on the mechanism which links transmitter release with presynaptic depolarization. Based on a series of experiments, Katz and Miledi (1967a, b, c, d) suggested that the depolarization opened a Ca gate (increasing calcium conductance) allowing this ion to enter the cell and reach the critical sites of ACh release.

Evidence that Ca combines with membrane release sites is found in experiments relating Ca to quantal release and in experiments investigating the competition between Ca, Na, and Mg. It has been
postulated that 3-4 Ca molecules are bound per binding site before release occurs (Hubbard et al., 1968b; Dodge and Rahaminoff, 1967). The membrane binding site for Ca has been linked to the adenosine 3'5' monophosphate (cyclic AMP) system (Rasmussen and Tenenhouse, 1968). The conversion of ATP, a strong chelator of Ca, to cyclic AMP, a weak chelator of Ca, by the enzyme adenylyl cyclase may increase the available Ca and facilitate release.

**Postsynaptic Membrane Phenomena:**

In 1958, Hodgkin suggested that when present in normal concentrations calcium ions are bound on the nerve membrane without changing the overall potential difference between the external and internal solutions and that in this state these ions influence the permeability and excitability of the membrane primarily by affecting the Na conductance. This explanation would also fit experimental data obtained with muscle fibers.

In the resting state, a muscle fiber is very sparingly permeable to calcium (Bianchi and Shanes, 1959). It follows that the resting membrane potential increases in high Ca concentrations (del Castillo and Stark, 1952; Jenerick and Gerard, 1959; Ishiko and Sato, 1957), whereas low Ca concentrations decrease the resting membrane potential producing depolarization (McIntyre, Young and Ware, 1956). In high external calcium concentrations, muscle membrane resistance
is increased (Tamashige, 1951; Jenerick, 1959). Furthermore, low calcium concentrations decrease the threshold (Jenerick and Gerard, 1953; Ishiko and Sato, 1957) and make the muscle fiber unstable (Bülbring, Holman, and Lullman, 1956).

An increased calcium concentration decreases the sensitivity of the subsynaptic membrane to ACh as evidenced by a decreased amplitude of the ACh potential (del Castillo and Engbaek, 1954; Takeuchi, 1963). Takeuchi (1963) demonstrated that an increase in extracellular Ca reduces the Na conductance of the endplate membrane produced by ACh with little or no effect on the potassium conductance. This evidence suggests that Ca may regulate membrane permeability by controlling Na conductance. Furthermore, Takeuchi (1963) found that the endplate membrane itself becomes permeable to Ca ion during ACh action. The physiological significance of the entrance of Ca into the cell during ACh action is not known, however, this may explain the local contractions of the muscle seen in the region of the endplate during iontophoretic application of ACh.

**Future Investigations**

Although much of the physiology of the neuromuscular junction has been illucidated through extensive research, many questions are as yet unanswered. For example, what is the mechanism through which quanta are released from the terminal's edge? What is the
function of Ca ion linking the depolarization produced by the nerve action potential with the release of quanta? What influence do the prostaglandins, hormones and the cyclic AMP system, found throughout the body, have on transmitter release or, perhaps, on the function of the subsynaptic membrane? and in what manner does ACh influence the receptor to induce a permeability change? Investigation into these unanswered questions alone will require years of research to uncover the complete story of the physiology of the neuromuscular junction.
CHAPTER I. INTRODUCTION

C. PHARMACOLOGY

In general, drugs may affect neuromuscular transmission at a variety of presynaptic and postsynaptic sites. Through various mechanisms drugs act at these sites to either facilitate or depress neuromuscular transmission. Important presynaptic mechanisms involve effects on: 1) transmitter synthesis, 2) the mechanism coupling ACh release with presynaptic depolarization through an action on Ca, 3) nerve terminal resting membrane potentials, 4) the amplitude and time course of the nerve action potential and 5) the release mechanism itself. Important mechanisms involved in postsynaptic drug actions include an effect on: 1) the subsynaptic receptor, 2) the passive membrane characteristics, and 3) the activated membrane characteristics.

Presynaptic Mechanisms

The drug, hemicholinium - 3 (HC-3) is known to block neuromuscular transmission presynaptically (Schueler, 1960) by depleting the nerve terminal of transmitter. The primary mechanism of action of HC-3 is to inhibit competitively the active transport system responsible for transporting choline to the site of ACh synthesis (Bowman,
1962). Neuromuscular blockade by HC-3 is slow in onset and develops when nerves are stimulated repetitively resulting in a progressive diminution in the amplitude of EPPS and MEPPS (Elmqvist and Quastel, 1965a). Triethylcholine is another drug which has pharmacological actions like those of HC-3 (Bowman and Rand, 1961; Bowman, Hemsworth, and Rand, 1962). In addition to its HC-3-like action, it is transported into the nerve terminals and is acetylated (Hemsworth and Smith, 1970); however, it has no ACh-like activity and does not act as a false transmitter (Bowman, Hemsworth, and Rand, 1962).

A toxin produced by Clostridium botulinum acts presynaptically to irreversibly inhibit transmitter release resulting in failures of EPPS (Burgen, Dickens, and Zatman, 1949), and curtailment of spontaneous release (Brooks, 1956). The ensuing paralysis appears to be due to a blockade of ACh release rather than to effects on depolarization-secretion coupling (Simpson, 1973). Hubbard and Quastel (1973) have suggested that the toxin may prevent the uptake of ACh by synaptic vesicles.

Changes in the presynaptic nerve action potential specifically modify evoked release. Tetraethylammonium causes an increase in the amplitude and duration of the action potential (Koketsu, 1958) by blocking K flux (Hagiwara and Saito, 1959) resulting in an increased evoked transmitter release without alterations in spontaneous release (Benoit and Mambrini, 1970). Conversely, certain local anesthetics,
e.g. procaine, selectively inhibit the Na conductance responsible for the presynaptic spike (Straub, 1956; Thesleff, 1956) and thus block release of transmitter from nerve terminals by abolishing the nerve action potential. The puffer fish poison, tetrodotoxin, has a similar mechanism of action (Narahashi, Moore, and Scott, 1964).

An alteration of the nerve terminal resting membrane potential is another important mechanism in considering drug effects. A facilitatory action produced by a reduction in extracellular Na ion concentration results in a small increase in MEPP frequency which is offset by a reduction of the amplitude of the presynaptic action potential (Gage and Quastel, 1966). This type of depolarization of the nerve terminal will increase spontaneous MEPP frequency; however, under conditions of stimulation, the depolarization will also reduce the amplitude of the nerve action potentials and thus reduce the magnitude of the evoked quantal release (Hubbard and Quastel, 1973). Compounds exhibiting these actions are the metabolic inhibitors, e.g. dinitrophenol, which inhibits the Na pump (Kraatz and Trautwein, 1957) and the steroid alkaloid, bactrachotoxin which blocks Na channels (Albuquerque, Warnick, and Sansone, 1971).

Apparently ACh itself also depolarizes the nerve terminal (Hubbard, Schmidt, and Yokota, 1965). An action of ACh on the nerve terminal was first proposed by Masland and Wigton (1940) and Feng and Li (1941) to account for the repetitive discharges or antidromic firing in
motor nerve after injections of acetylcholine or anti-ChEs. The effect is small since in the presence of prostigmine, acetylcholine decreases the quantal content of the EPP (Ciani and Edwards, 1963; Hubbard, et al., 1965) but does not affect MEPP frequency (Fatt and Katz, 1952; Hubbard, et al., 1965). Hubbard, et al. (1965) proposed that the site of depolarization in this case may be the first node of Ranvier, but that depolarization of this site would probably be insufficient to alter ACh release. Hubbard (1965) concluded that ACh does not have an essential physiological role in the mechanism of transmitter release at the neuromuscular junction.

Other facilitatory drugs may produce repetitive antidromic firing which cannot be correlated with acetylcholinesterase inhibition (Riker, Roberts, Standaert, and Fujimori, 1957). For example, succinylcholine (SCh) was found to elicit antidromic firing (Standaert and Adams, 1965). Blaber and Karczmar (1967a) suggested that SCh may act at the same presynaptic site as ACh, i.e. the first node, since Blaber (1967) did not observe an increase in MEPP frequency with SCh, necessary if the nerve terminal were depolarized. Anti-ChE's similarly have been shown to have little effect in increasing MEPP frequency in cat muscle (Boyd and Martin, 1956a; Blaber and Christ, 1967), and no effect in the frog (Fatt and Katz, 1952) or in the rat (Liley, 1956a); in the cat, however, quantal content was increased by these agents (Christ, 1969). Werner (1960), Hubbard and Schmidt
and Blaber (1972) have suggested that the mechanism of antidromic firing produced by many facilitatory drugs may be the result of augmentation and/or prolongation of the negative after potential of the nerve action potential which subsequently re-excites the first node.

It has been postulated that d-tubocurarine (dTc) may also act presynaptically and may itself depolarize (Hubbard, Schmidt, and Yokota, 1965). DTC has been reported to have no effect on transmitter release from nerve terminals (Dale, Feldberg and Vogt, 1936; Krnjevic and Mitchell, 1961; Beranek and Vyskocil, 1967; Bowen and Merry, 1969; Fletcher and Forrester, 1970; and Auerbach and Betz, 1971); however, there are also investigations reporting that dTc reduces the amount of transmitter released (Lilleheil and Naess, 1961; Beani, Bianchi, and Ledda, 1964; Standaert, 1964; Riker and Okamoto, 1969; Blaber, 1970; and Galindo, 1971).

Using the transversally cut muscle fiber preparation, Hubbard, Wilson, and Miyamoto (1969) observed that in the rat dTc increased probability of release and decreased mobilization thereby depleting the available store of transmitter. In the cat, similar interpretations were suggested (Blaber, 1970; Jacobs and Blaber, 1971). Auerbach and Betz (1971) criticized the findings of Hubbard, et al. (1969) on the basis that in the cut muscle fiber preparation the endplate regions could not be located due to an increase in the membrane space constant
associated with the operative procedure. Because of this altered space constant, they suggested that the recordings in a cut fiber are made from a remote location yielding inaccurate estimates of EPP amplitudes. Hubbard and Wilson (1973) observed that the altered space constant reported by Auerbach and Betz (1971) was, in fact, in the range reported for normal uncut rat diaphragm muscle (Zolonick, Norman, and Fedde, 1970). In their study the postsynaptic effect of dTc appeared at a lower concentration than the presynaptic effect. A presynaptic effect was observed in the cat in the concentration normally used to block muscle contraction (Blaber, 1970; Jacobs and Blaber, 1971). Blaber (1970) suggested that dTc may exert an action at the cholinceptive site on the unmyelinated terminal.

An effect of a drug on the quantal content of EPPS and MEPP frequency in parallel indicates an action on the process which couples release to polarization (Hubbard, Llinas, and Quastel, 1969). The increase in quantal content of the EPP and an increase in MEPP frequency produced by increasing Ca concentrations (Boyd and Martin, 1956a, b; Jenkinson, 1957; Hubbard, 1961; Hubbard, Jones, and Landau, 1968a, b) is considered a good indication that the ion is involved in the mechanism which links release with presynaptic depolarization (Katz and Miledi, 1967a, b, c, d). The nature of the neuromuscular block caused by the antibiotic neomycin is similar to that of Ca lack; the quantal content of the EPP is reduced and the
acceleration of MEPP frequency by K inhibited. These effects were reversed by an increasing Ca concentration (Elmqvist and Josefsson, 1962).

Phenolic substances, phenol and catechol, increase the quantal content of EPPS (Otsuka and Nonomura, 1963; Kuba, 1969; Gallagher and Blaber, 1973) and increase the frequency of MEPP (Kuba, 1969; Gallagher and Blaber, 1973). Ouabain and other cardiac glycosides block active transport of Na and cause an accumulation of intracellular Ca, resulting in an increased quantal content and MEPP frequency (Birks, 1962, 1963; Birks and Cohen, 1968a, b).

Elevation of extracellular K ion concentration results in an increase in quantal content of the EPP (Takeuchi and Takeuchi, 1961; Edwards and Ikeda, 1962) and increased frequency of MEPPS (Liley, 1956c; Gage and Quastel, 1965; Hubbard, et al., 1965); an increase in the available store has also been reported (Parsons, Hofmann, and Feigan, 1965). Thesleff and Quastel (1966) suggested that K may effect depolarization-release coupling.

A direct effect of a drug on transmitter release is one that occurs independently of a change in presynaptic membrane potential or permeability of Ca (Hubbard and Quastel, 1973). Ethanol exerts this direct type of facilitatory action (Gage, 1965; Quastel, Hackett, and Cooke, 1971).
Postsynaptic Mechanisms

Compared to nerve terminal action, little is known about the underlying mechanisms of drugs acting postsynaptically. The effects of the majority of the drugs can be attributed to an association with the ACh receptor molecule. This combination may or may not lead to ionic permeability changes resulting in a depolarization and/or blockade.

The best known example of a non-depolarizing receptor blocking drug is d-tubocurarine. The classical experiments of Claude Bernard (1856) localized the blocking action of curare at the junction between nerve and muscle. Under the influence of dTc, the EPPS diminish in size until they fail to reach the critical level necessary to initiate a muscle action potential. Studies have shown that dTc does not alter the resting membrane potential or electrical properties of the muscle membrane (del Castillo and Katz, 1957). On the activated subsynaptic membrane, dTc has been shown to decrease the magnitude of the conductance change produced by ACh but does not alter the ratio of the specific ionic conductances (Takeuchi and Takeuchi, 1960b). The paralysis is reversed by procedures which increase the local concentration of ACh.

Anticholinesterase drugs, through the prevention of enzymatic hydrolysis, permit the ACh depolarization to persist. The result is an increase in the amplitude and duration of MEPPS (Blaber and Christ,
When the MEPPS summate, they sometimes trigger action potentials and produce spontaneous twitches in single muscle fibers; EPPS are affected in a similar manner (Fatt and Katz, 1955; Boyd and Martin, 1956a, b). The prolonged EPP caused by anti-ChEs may act as a persistent current "sink" and result in the generation of trains of muscle action potentials (i.e., repetitive firing).

A drug-receptor interaction which produces a depolarization is a more complex phenomenon. The interaction between the depolarizing agents and the receptor produces an initial depolarization of the endplate. If the contact is prolonged, a reduction in the sensitivity of the endplate region to the transmitter occurs producing receptor desensitization and blockade. The persistent depolarization of the muscle fiber results in inactivation of the Na-carrying mechanism responsible for generation of the action potential (Thesleff and Quastel, 1966). Thus endplate potentials are reduced in amplitude due to the depolarization and are unable to elicit action potentials due to inactivation of the spike generating system. ACh itself in high concentrations is known to produce a similar neuromuscular paralysis (Bacq and Brown, 1937). Typical depolarizing agents are carbachol, choline, succinylcholine, and decamethonium. Depending on the species, depolarizing agents may exhibit a dual mode of action (Zaimis, 1953); i.e., the depolarizing action is followed by a block characteristic of that produced by dTc. Churchill-Davidson and
Richardson (1952) reported that muscles of myasthenia gravis patients were extremely resistance to decamethonium ($C_{10}$) in which the paralysis is of the dual type. The difference in type of block produced by these agents exists between muscles of different species and among different muscles of the same species. Carbachol and $C_{10}$ have been shown to alter the ionic conductances in the same manner as ACh since the reversal potential of these responses does not differ from that of ACh (Feltz and Mallart, 1971b).

Another phenomenon which may be species dependent is sensitization (Koketsu and Gerard, 1956; Karczmar, 1957; Kim and Karczmar, 1967; Karczmar, Nishi, and Blaber, 1972). This phenomenon is defined pharmacologically as a facilitatory postsynaptic action of certain agents such as oxamides, hydroxyaniliums and NaF. These agents exhibit presynaptic actions, inhibit cholinesterase, and antagonize both depolarizing and non-depolarizing blockers; however, these actions are distinct from their postsynaptic sensitizing effect (Koketsu and Gerard, 1956; Karczmar, 1957; Karczmar, Kim, and Blaber, 1965; Koketsu, 1966; Karczmar, Koketsu and Soeda, 1968). This phenomenon is most noticable in amphibia (Karczmar, Kim, and Koketsu, 1961; Karczmar, et al., 1965, 1968); however, a sensitizing component of the action may also be present in the cat (Jacobs and Blaber, 1969).

Karczmar, et al., (1968) proposed that the sensitizing action theoretically may consist of an effect on the postsynaptic cholinergic
receptor involving a change in the configuration. Under these conditions the response to ACh may be enhanced. Since sensitizers have no effect on the resting membrane potential, only the response to the membrane activated by ACh is involved.

It is obvious that a drug can exert actions on a multiplicity of sites. Blaber and Karczmar (1967b) postulated at least five cholinceptive sites at the neuromuscular junction of the cat: 1) the motor endplate, 2) acetylcholinesterase, 3) the motor nerve terminal, 4) the first node of Ranvier, and 5) the choline carrier system. Drugs affecting more than one site may possess both facilitatory and neuromuscular blocking actions, such as methoxyambenonium (Karczmar, 1957; Blaber and Christ, 1967).

Adrenergic Sites

Non-cholinceptive sites of action at the neuromuscular junction have also been proposed (Bowman and Raper, 1965). Bowman and Raper (1967) postulated that adrenergic α receptors are present in the motor nerve ending increasing transmitter release, while β receptors in the muscle fiber are associated with hyperpolarization of the muscle membrane. Using electrophysiological microelectrode techniques, Kuba (1970) found that at the rat neuromuscular junction 1) noradrenaline acts primarily on the nerve terminal increasing the release of transmitter, 2) isoproterenol acts solely on the muscle
membrane increasing the input resistance and hyperpolarizing the membrane and 3) adrenaline has both pre- and postsynaptic actions. Thus, all autonomic transmitters, acetylcholine, adrenaline and noradrenaline, may have a role in neuromuscular transmission.

It is obvious from this review of the pharmacology of the neuromuscular junction that a drug may act at a number of sites. However, other possible sites of drug action may not as yet be defined. The purpose of this study is to investigate the sites of action of theophylline and to explore concurrently novel sites of drug action.
CHAPTER I. INTRODUCTION

D. THEOPHYLLINE

Theophylline or Theocin is a naturally occurring constituent of tea. Tea leaves prepared from *Thea sinensis* contain two alkaloids: caffeine, in a concentration of one to four percent and theophylline in a very small percentage. Theophylline or 1, 3 dimethylxanthine was first isolated from tea in 1885 (Claus and Tyler, 1965). It was synthesized by Traube in 1900 from dimethylurea and ethylcyanoacetate (Stecher, 1968). The structure is shown in Figure 3. Since the drug is only slightly soluble, it is combined with compounds which render it more soluble. For this reason, the ethylenediamine salt of theophylline was used in this study. Cornish and Christman (1957) found that in man theophylline is excreted primarily as a mono- (3-methyl and/or 1-methyl) uric acid or as a 1, 3-dimethyl uric acid.

**Biochemical Action**

Theophylline has recently been brought into prominence due to its interaction with the biochemical hormone regulator, adenosine 3'5' monophosphate (cyclic AMP). Methylxanthines and particularly theophylline inhibit the enzyme phosphodiesterase which is responsible for breaking down cyclic AMP (Butcher and Sutherland; 1962). Phos-
Figure 3. Structure of theophylline (1, 3dimethyl-xanthine).
phodiesterase is found widely distributed in all mammalian tissues examined with the exception of red blood cells (Drummond and Perrott-Yee, 1961). By inhibiting this enzyme, methylxanthines elevate the tissue level of cyclic AMP and exaggerate the effects of the expected response to a hormone; indeed, the response to the methylxanthines has been taken as positive evidence that cyclic AMP is a mediator of hormone action (Butcher, 1968). The diagram below illustrates the basic reactions:

\[
\begin{align*}
\text{adenyl cyclase} & \quad \downarrow \\
\text{ATP} & \rightarrow \text{CYCLIC AMP} \\
\text{phosphodiesterase} & \quad \downarrow \\
\text{5'AMP} & \rightarrow \\
\end{align*}
\]

Elevation of cyclic AMP is also accomplished by stimulation of the enzyme adenyl cyclase. Catecholamines possessing \( \beta \) adrenergic activity and hormones such as corticotropin are known to activate adenyl cyclase which some investigators believe is the \( \beta \) adrenergic receptor (Robison, Butcher, and Sutherland, 1969).

**Metabolic Action**

Cyclic AMP accumulation in the liver cell increases glycogenolysis by stimulating dephosphophosphorylase kinase. This effect of cyclic AMP is potentiated by xanthines. A diminished glucose
uptake has been measured in adipose tissue indicating an anti-insulin action (Rodbell, 1967) in response to theophylline. Thus a synergism exists between theophylline, cyclic AMP and catecholamines in producing hyperglycemia (Northrup and Parks, 1964; Triner and Nahas, 1966).

Inhibition of phosphodiesterase also produces an activating effect on lipase enzymes which mobilize free fatty acids from adipose tissue into the plasma (Triner and Nahas, 1966). An enhanced response occurs in the presence of adenyl cyclase stimulators, such as catecholamines or ACTH (Weiss, Davies, and Brodie, 1966).

**Potentiation of Hormonal Action**

Additional drug effects of theophylline have become apparent due to the potentiation of cyclic AMP in hormonal systems. Phosphorylase activation and insulin release in response to glucagon and enhanced steroidogenesis in response to ACTH have all been potentiated by methylxanthines (Sutherland, Robison, and Butcher, 1968). Similarly, the action of vasopressin on urinary bladder permeability (Handler, Butcher, Sutherland, and Orloff, 1965) and the action of parathyroid hormone on serum calcium (Wells and Lloyd, 1967) are potentiated by theophylline. Thyroid hormone production in response to thyroid stimulating hormone and melanocyte dispersion in response to melanocyte stimulating hormone are also enhanced by methylxanthines.
(Sutherland, et al., 1968). Finally, iodine trapping in the thyroid gland in response to thyrotropin (Bastomsky and McKenzie, 1968) and HCl production in the gut (Sutherland, et al., 1968) are linked to cyclic AMP, as they too are potentiated by methylxanthines.

Although theophylline responses usually fit nicely into the cyclic AMP picture, there are some discrepancies. Sattin and Rall (1970) reported that theophylline inhibited rather than potentiated, an adenosine-induced increase in cyclic AMP in rat cerebral slices. In rabbit cerebral cortex or cerebellum prepared at room temperature, methylxanthines do not augment cyclic AMP levels (Kakiuchi, Rall, and McIlwain, 1969); however, when prepared in the cold, theophylline increased cyclic AMP concentration (Kakiuchi and Rall, 1968a, b). At the adrenal cortex, the glycolytic action of ACTH is also inhibited rather than potentiated by theophylline (Birmingham and Bartova, 1973). Thus, the action of theophylline does not always potentiate the cyclic AMP system. Therefore, caution must be exerted before assigning a role for cyclic AMP in a response based solely on the effect of theophylline.

**Action on Enzymes**

Aside from an inhibition of phosphodiesterase, theophylline has proved to be a cholinesterase inhibitor (Bounameaux and Goffart, 1949; Vincent and Lagrere, 1950; Bose, Vijayvargiya, and Saifi, 1960). Vincent and Lagrere (1950) showed that both theophylline and theo-
phylline ethylendiamine in a concentration of \(2 \text{ mM}\) produced a 20% inhibition of acetylcholinesterase obtained from sheep brain but had no effect on pseudocholinesterase from horse serum. Using human red blood cells and serum, theophylline produced a 66% inhibition of acetylcholinesterase and 32% inhibition of pseudo-cholinesterase in a concentration of \(2 \text{ mg/ml} \ (0.11 \text{ M})\) (Bose, et al., 1960). Thus, in reviewing the literature, it appeared that theophylline's action on enzymes was limited to inhibition of phosphodiesterase and cholinesterase.

**Central Nervous System**

The stimulant action of the methylxanthines on all portions of the CNS is well known. Caffeine is the most potent while theophylline is less so with theobromine having the least action. Evidence suggests that the initial site of this action is on the cerebral cortex, as an EEG arousal pattern is produced (Toman and Davis, 1949), and cortically evoked action potentials, produced by thalamic stimulation, are also increased (Gerber, 1961). At the spinal cord level, caffeine has also been shown to facilitate polysynaptic rather than monosynaptic reflexes in cats (Sant'Ambrogio, Frazier, and Boyarsky, 1962).
Heart

Theophylline stimulates the myocardium to produce a positive chronotropic and inotropic effect. It is the most potent cardiac stimulant of the methylxanthines. The exact mechanism by which theophylline enhances myocardial contractility is unknown. Several hypotheses have been proposed: an increased uptake of Ca (De Gubar-eff and Sleator, 1965; Guthrie and Nayler, 1967), and an increase in the intracellular level of cyclic AMP (Sutherland, et al., 1968). The cyclic AMP hypothesis is supported by experiments using electrically driven atria in which the inotropic effect of norepinephrine is potentiated 8-fold by the addition of 1 mM theophylline (Rall and West, 1963). At low stimulation frequencies, caffeine increased the duration of the transmembrane action potential, similar to a catecholamine action (De Gubareff and Sleator, 1965); while at high stimulation frequencies, the action potential rises faster and is shortened resembling the effect of calcium (Shibata and Hollander, 1967). The action of theophylline may also have a sympathomimetic component in its effect on the heart by evoking endogenous catecholamine release (Westfall and Fleming, 1968). Marcus, Skelton, Grauer, and Epstein (1972) showed that this amounted to 40% of the contractile response.

The mechanism responsible for the noncatecholamine component of the inotropic effect of theophylline may be attributed to inhibition
of phosphodiesterase, since imidazole, a stimulant of phosphodiesterase activity, reduces the increased contractility induced by the xanthines but not by catecholamines (Kukovetz and Poch, 1967). In contrast, the increase in the duration of the active state of the papillary muscle preparation in response to theophylline does not mimic the cyclic AMP effect, since norepinephrine, glucagon, and dibutyryl cyclic AMP, decrease the duration of the active state (Epstein, Levey, and Skelton, 1971; Skelton, Levey, and Epstein, 1970).

Another means through which theophylline may exert its cardiac effects is by changing intracellular ionized calcium concentration. Cardiac muscle contraction is believed to be initiated by an increase in the concentration of ionized calcium available in the vicinity of the contractile apparatus. Several studies suggest that methylxanthines alter myocardial contractility by altering calcium kinetics. For example, caffeine increases the rate of Ca influx and efflux from cardiac muscle (Nayler, 1963) and the rate at which cardiac mitochondria release calcium, while decreasing the rate at which cardiac mitochondria accumulate Ca (Nayler and Hasker, 1966); calcium uptake by glycerinated cardiac slices is also partially inhibited (Fairhurst, Palus, and Jenden, 1967). Similarly, an adenosine-induced heart block, which has been attributed to an interference with calcium uptake, is reversed by caffeine and accompanied by an increase in
calcium accumulation (De Gubareff and Sleator, 1965; Guthrie and Nayler, 1967). Thus, theophylline may stimulate cardiac contracility through several mechanisms: catecholamine release, increased cyclic AMP levels, or increased calcium flux.

**Smooth Muscle**

Theophylline exerts a relaxing effect on the smooth muscle of the bronchioles, the coronary, pulmonary, renal, and peripheral blood vessels, and the gastrointestinal tract; however, actions of the xanthines not related to a direct smooth muscle effect may modulate these phenomena. Peripheral vessels are dilated by xanthines but the stimulation of the vasomotor system in the brain stem produces a pressor action which antagonizes the effect (Hahn, 1960). Diuresis is a result of both increased cardiac output and increased renal blood flow (Davis and Shock, 1949). Similarly, bronchiolar relaxation is facilitated by pulmonary vasodilation and the stimulation of the respiratory center also produced by theophylline (Le Messurier, 1936). Theophylline is more potent than caffeine as a bronchodilator (Macht and Ting, 1921). This bronchodilator effect has been applied to the treatment of asthma (Segal, Levinson, Bresnick, and Beakey, 1949).

The site of theophylline action follow the same distribution as \( \beta \) adrenergic receptors. In the gut, however, the role of the \( \beta \)
receptor is still controversial. Robison, Butcher, and Sutherland (1967) suggest the possibility that an increase in the cyclic AMP content (β receptor stimulation) produces relaxation and decreased contraction. However, the experiments of Ito and Kuriyama (1971) do not suggest that cyclic AMP is the mediator of the effects of theophylline on the gut.

Ito and Kuriyama (1971) classified the action of caffeine on the gut into three phases: 1) initial slow contraction phase, 2) tension development, and 3) relaxation phase. They showed that the first stage is sodium independent, and that the second stage is sodium dependent accompanied by an increased frequency of spikes. Furthermore, the third phase is accompanied by depolarization block of the spike followed by relaxation, whereas block after β receptor stimulation has been associated with membrane hyperpolarization. Ito and Kuriyama (1971) suggested that caffeine produced these effects by mobilizing the bound calcium from the membrane and increasing sodium permeability. Although the effect on the gut is of minor therapeutic importance in comparison to its other actions it lends supportive evidence, by virtue of caffeine's Ca mobilizing effect, to the actions of theophylline on skeletal muscle.

**Skeletal Muscle**

Methylxanthines have long been known to have a direct action on
skeletal muscle. The enhanced contractility of frog muscle by caffeine is a function of concentration. At a concentration of 1 mM, caffeine potentiates directly stimulated muscle contraction, prolongs the active state, and reduces the threshold of a potassium-induced contracture (Sandow, Taylor, Isaason, and Seguin, 1964). At concentrations of 2-5 mM, caffeine causes a graded contracture and prolongs a potassium contracture, which is reversible when caffeine is removed from the media (Axelsson and Thesleff, 1958; Bianchi, 1961). In higher concentrations above 5 mM, caffeine produces irreversible rigor. In contrast to caffeine, there is no available data on the effects of theophylline on muscle contracture as a function of concentration; however, Roman and Jacobs (1973) reported that at equimolar concentrations (5 mM) theophylline produced a contracture equal in intensity to that of caffeine.

Bianchi (1961) demonstrated that a marked influx and efflux of calcium ions accompanies the caffeine contracture. In addition, Axelsson and Thesleff (1958) showed that: 1) the contracture could occur independently of membrane depolarization since it was produced in a muscle completely depolarized by K$_2$SO$_4$; and 2) caffeine acted on the outer surface of the muscle membrane since injection into the muscle was without effect. Based on these findings, Bianchi (1961) suggested that caffeine reduced the binding of calcium to sites in or on the muscle membrane. Weber and Herz (1968) also found
that caffeine caused a release of calcium from the isolated sarcoplasmic reticulum. These findings have been substantiated on fragmented sarcoplasmic reticulum from which theophylline also was found to increase the rate and extent of calcium efflux (Johnson and Inesi, 1969).

In 1968, Bianchi suggested that the methylxanthines may mobilize stored or bound calcium which together with the calcium released by the action potential leads to a supranormal level of internal free calcium and enhancement of myofibrillar contraction. Thus, in intermediate concentrations, the calcium released by caffeine would be sufficient to cause a contracture, and, at very high concentrations, an irreversible inhibition of calcium uptake by the reticulum. Using electronmicrography of precipitated stained calcium ions, McAlister (1972) observed this phenomenon and indicated that calcium release also took place in the terminal cysternae of the transverse tubular system.

Prior administration of local anesthetics (e.g. procaine and tetracaine) block the caffeine contracture and associated efflux of calcium in frog sartorius muscle (Feinstein, 1963). Similar results have been obtained with theophylline treatment in isolated fragmented sarcoplasmic reticulum (Johnson and Inesi, 1969). It is thought that the local anesthetics may act by preventing methylxanthines from releasing calcium from binding sites in muscle.

Aside from changes in muscle contractility, methylxanthines are
known to stimulate muscle metabolism (Gasser, 1930), and to produce an increase in the oxygen consumption of muscle (Gemmill, 1947). This effect is blocked by the removal of external calcium (Yabu, 1963). Theophylline has also been shown to potentiate the activation of phosphorylase a by epinephrine (Hess, Hottenstein, Shanfeld, and Haugaard, 1963). Calcium activates phosphorylase b kinase, needed to activate phosphorylase a in skeletal muscle (Kreb, Graves, and Fischer, 1959); and both excess calcium ions and theophylline treatment also facilitate contraction of diaphragms depressed by excess potassium (Bowman and Raper, 1964). Thus, some of the evidence suggests that the increased metabolism and increased contractility of muscle produced by theophylline may be related.

Although it has not yet been demonstrated conclusively it is likely that the cyclic AMP system may be involved in the function of skeletal muscle. Cyclic AMP levels have been shown to be present and increased by epinephrine (Posner, Stern and Kuhs, 1965). Bowman and Nott (1969) postulated that in white fast contracting muscle, the cyclic AMP system either is involved in the release of calcium from the sarcoplasmic reticulum or acts as a brake on rebinding. By increasing levels of cyclic AMP, β receptor stimulation increases the levels of free calcium in the region of the myofilaments, and thereby prolongs the active state. Thus this mechanism may be involved in the effect of theophylline on muscle contraction.
Neuromuscular Transmission

Huidobro (1945) first reported that theophylline had a facilitatory effect on neuromuscular transmission. In addition to a peripheral antifatigue effect, he observed that theophylline possessed a decurarizing action on cat quadriceps femoris muscle stimulated indirectly at various frequencies. The effect of theophylline at low frequencies summated with the facilitation due to prostigmine. The author attributed the facilitation to a lowering of the excitatory threshold of acetylcholine at the level of the neuromuscular junction, a postsynaptic effect.

In 1945, Torda and Wolf demonstrated that theophylline in a concentration of 0.2 mg/ml (1.1 mM) enhanced an acetylcholine induced contracture of frog rectus abdominis muscle, characteristic of acetylcholinesterase inhibition.

Experiments by Goutier (1949a) showed that theophylline sensitized the frog rectus to potassium; in contrast, this author suggested that the effect was not due to the anticholinesterase activity of the methylxanthines. In another study, Goutier (1949b), reported that theophylline did not modify the action potential of the isolated frog sciatic nerve; yet there was an increase in the action potential of indirectly stimulated frog sciatic nerve-sartorius muscle preparations which was reversible. A weak anti-curare effect was also demonstrated and, even after complete curarization, theophylline potentiated
the response to direct muscle stimulation.

Walther (1961) found that in normal calcium solution an increase in potassium ion concentration potentiated theophylline's action. Changes in Ca ions, as well as in the K:Ca ratio of the bathing solution did not significantly influence the action of theophylline. Walther (1962) suggested that the increased efficiency produced by theophylline in preparations fatigued by high frequency stimulation was due to an improved functioning of the endplates.

It should be noted that in all investigations up to this period the effects of theophylline on neuromuscular transmission were all attributed to a postsynaptic site of action at the endplate.

In 1967, Breckenridge, Burn, and Matschinsky demonstrated that a 100 mg total dose of theophylline potentiated the facilitation produced by epinephrine and neostigmine on the in vivo cat sciatic gastrocnemius preparation. These effects of theophylline were not evident in a curarized preparation stimulated directly; nor was any facilitation produced in indirectly stimulated preparations when this same concentration of theophylline was administered alone. These authors concluded that the facilitation of contraction by theophylline was due solely to an increased level of cyclic AMP within the nerve terminal augmenting acetylcholine release presynaptically producing repetitive firing of muscle units. In attempting to reproduce these effects in our laboratory, Blaber and Gallagher (1971) found that a
25 mg/kg dose of aminophylline produced a 30% facilitation of muscle contraction.

Based on the conclusion of Breckenridge, et al. (1967), Goldberg and Singer (1969) investigated the effects of theophylline on the isolated rat phrenic nerve-diaphragm preparation using intracellular microelectrode techniques. The amplitude of endplate potentials was increased in curarized preparations. A similar facilitatory action of theophylline was produced in low Ca - high Mg blocked muscle in which EPP quantal content and MEPP frequency were increased. These effects were again attributed to an inhibition of phosphodiesterase by theophylline increasing levels of cyclic AMP presynaptically.

In an attempt to elucidate the mechanism of action of theophylline on the cyclic AMP system in skeletal muscle, Varagic and Zugic (1972) studied the interaction of theophylline with other drugs involved in the cyclic AMP system using the in vitro rat phrenic nerve-diaphragm preparation. Theophylline produced an augmentation of muscle contraction caused by either direct or indirect stimulation. These effects were abolished by nicotinic acid, a stimulator of phosphodiesterase activity. Theophylline was also shown to have an anti-dTc effect. The augmentation of muscle contraction and anti-dTc effect were potentiated by norepinephrine and neostigmine. The authors concluded that these effects were due to an increase in cyclic AMP levels presynaptically, increasing ACh release and also directly
on the muscle increasing contractility.

**Blood Levels**

An intravenous dose of 0.5 grams theophylline produces an immediate blood level of 1.2 mg/100ml; diuresis and bronchodilation are estimated to occur at blood levels of 0.5 to 1 mg/100 ml (Truitt, McKusick, and Krantz, 1950). The neuromuscular junction, however, appears to be more resistant to the actions of theophylline. *In vivo* doses of 100 mg are required to produce neuromuscular facilitation in the cat (Breckenridge, et al., 1967). Similarly, a dose of 1.8 to 2.4 gm/day is needed to effect neuromuscular function in myasthenia gravis patients (Brumlik, Jacobs, and Karczmar, 1973). *In vitro* studies, the concentration ranged from 0.378 mg/ml to 1 mg/ml; this range produced neuromuscular facilitation in normal and myasthenic human muscle samples (Jacobs, Karczmar, and Brumlik, 1973).

**Theophylline and Myasthenia Gravis**

Based on the facilitatory effects of phosphodiesterase inhibitors (Breckenridge, et al., 1967). Jacobs, Brumlik, and Karczmar (1971) studied the effects of theophylline on biopsy samples obtained from a patient diseased with myasthenia gravis who was refractory to anticholinesterase therapy. *In vitro* theophylline restored muscle fatigue; a tetanus, at a frequency of 30 per second was sustained
while during the control period the muscle progressively weakened in response to a 3 per second stimuli (Jacobs, et al., 1971, 1973). Clinically, theophylline was found to facilitate muscle contraction of myasthenia gravis patients refractory to anti-cholinesterase therapy (Brumlik, et al., 1973).

The disease myasthenia gravis was described by Jolly (1895) as a failure of the muscle response to repetitive indirect stimulation but not to direct stimulation. Thus the primary defect in the disease appears to be in the transmission process. There are three current theories regarding this disorder of neuromuscular transmission:

1) transmission phenomenon, 2) improper anatomical configuration, and 3) an auto-immune phenomenon.

Electrophysiological studies on myasthenic muscle in vitro revealed that there are small MEPPS present that may result from a presynaptic defect in ACh synthesis (Elmqvist, Hofmann, Kugelberg, and Quastel, 1964). This defect in quantum size (MEPP amplitude) is not apparently due to an inhibition of ACh synthesis like that produced by hemicholinium-like drugs since the small MEPPS do not respond to treatment with choline.

This investigation of the effects of theophylline on neuromuscular transmission was based on the fact that theophylline improved muscle contraction in some patients diseased with myasthenia gravis (Jacobs, et al., 1971;
1973), and that the primary defect in the disease appeared to be associated with the neuromuscular transmission process (Jolly, 1895). In addition, an attempt was made to resolve the apparent conflict in the literature regarding the pre- versus postsynaptic effect of theophylline at the neuromuscular junction.
CHAPTER II

METHODS
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METHODS

Mongrel cats of either sex, weighing between 1.5-5.0 kg were used in all experiments. The animals were anesthetized with a mixture of $\alpha$-chloralose, 60 mg/kg, pentobarbital, 5 mg/kg and atropine, 1.5 mg/kg, injected intraperitoneally. Surgical anesthesia was produced in 30-40 minutes with a duration of up to 24 hours. All cats, however, were sacrificed at the end of each day's experiment.

Surgical Procedure

An incision through the skin is made from the Achilles tendon to above the biceps femoris along the popliteal space. The skin is removed with a cautery, exposing the lower part of the tenuissimus which lies in the popliteal space. The biceps is carefully bisected with the cautery tip to expose the upper tenuissimus which adheres to its fascia. Simple dissection from the connective tissue allows the tenuissimus to be viewed completely. Care must also be exerted to prevent injury to its small nerve which arises perpendicularly from the sciatic. The muscle is freed, proximally and distally, and the small nerve ligated after dissection from the sciatic.

The nerve-muscle preparation is placed in an isolated chamber
and perfused with oxygenated Krebs-Henseleit Ringer solution pre-warmed to 37°C (Boyd and Martin, 1956a). Once in the isolation chamber, the muscle is cleaned of its adhering connective tissue under a low power microscope using microsurgical tools.

The entire surgical procedure and dissection take approximately 45 minutes.

**Muscle Chamber**

The muscle bath consisted of plexiglas, 9.0 cm x 1.2 cm, with a fluid level of 5 mm, yielding a volume of 5.4 ml. Two 500 ml leveling resevoirs placed 105 cm above the bath contained the nutrient solution which was aerated with a mixture of 95% O₂ and 5% CO₂. The resevoirs - one containing control Ringer solution and the other containing test drugs dissolved in Ringer solution - were filled to equal volume. Rubber tubing, 3.0 mm I.D., connected the resevoirs to a common inlet where the flow rate was regulated. The solution passed from the common inlet through polyethylene tubing (P.E.) (.043 in. I.D.) coiled in a heated water jacket which maintained the perfusion temperature at 37 ± 0.2°C.

The P.E. tubing was attached to an 18 G needle and formed the inflow unit of the muscle bath. The flow rate was adjusted to 10 ml/min; thus, the time for complete turnover of the bath volume was about 34.0 sec.
The muscle was placed across a piece of plexiglas located in the center of the chamber and pinned on both sides to balsa wood. This arrangement allowed transilluminated viewing with an American Optical microscope (maximal magnification 107.5 X).

The nerve innervating the muscle was pulled through a small opening into an adjacent isolation chamber containing bipolar platinum electrodes. The isolation chamber reduced the stimulation artifact while permitting limited perfusion of the nerve.

Recording System

The recording system was similar to that described by Fatt and Katz (1951) and Boyd and Martin (1956a). To record electrical events at the endplate region of the muscle microelectrodes were employed. Glass microelectrodes were prepared, using a David Kopf vertical capillary puller. The resistance was estimated by the rate of ethanol flow into the tip of the capillary; electrodes prepared in this manner have been shown to have a tip diameter of 0.5 to 1.0 μm and resistance of 4-25 MΩ. The microelectrodes were filled under vacuum with ethyl alcohol which was then displaced with distilled water and finally with 3M KCl.

Microelectrodes were mounted on a Narishige micromanipulator. A Ag-AgCl wire was placed inside the pipette and connected to a DC amplifier. The Bioelectric NFl DC amplifier recorded the potential
difference between the glass electrode inside the cell and indifferent Ag-AgCl electrode placed in the physiological salt solution. To accurately measure the amplification of the system, a Bioelectric CA 5 calibrator was introduced into the recording system. The output of the DC amplifier was further amplified and displayed on two monitor oscilloscopes - a Tektronix RM 561A and a Tektronix RM 564. The RM 561A was used to view resting membrane potentials; the RM 564 at a higher amplification served to monitor the smaller electrical events. A 502A Tektronix dual beam oscilloscope with an even higher sensitivity was used to display the data which was recorded on 35 mm film with a Nihon Kohden Reflex PC-4 camera. The film was then placed in a Standard viewgraph and the data measured.

**Stimulating System**

The nerve was stimulated using a Grass S88 stimulator through an SIU 5 stimulus isolation unit (see Figure 4). Square wave pulses (50-100μsec duration) were employed. In experiments involving evoked potentials threshold voltage was determined on each preparation as the lowest voltage needed to elicit a muscle twitch. This voltage was then doubled to approximate maximal orthodromic stimulation and then doubled again to insure supramaximal stimulation.
Figure 4. Diagram of the stimulating and recording systems used in single intracellular microelectrode studies. Bath temperature is maintained at 37°C; magnification = 107.5 X. Abbreviations are described in the text.
MEPP and EPP Recording

Microelectrodes were inserted at the endplate region of the muscle fiber where spontaneous MEPPS of shortest rise-times and greatest amplitudes were recorded. Only those cells having MEPPS with rise-times of less than 1 msec and 1/2 falls of less than 1.5 msec were acceptable. Each cell was held for a ten minute control period which allowed the resting membrane potential to stabilize at -70 mV or greater. If the resting membrane potential declined by greater than 5 mV during this period, the cell was rejected. The MEPPS were displayed at a 1 cm/sec sweep on the oscilloscope. Thirty one second sweeps were filmed on the camera in a vertical continuous mode at .5 cm/sec.

With this recording arrangement, both MEPP frequency and amplitude could be obtained. These parameters were measured in the same fiber ten minutes after treatment with theophylline. The data collected during the last one minute of the ten minute control period was compared to the data obtained during the last one minute of the treatment period in this and all subsequent experiments. A paired statistical analysis was performed comparing the mean control response with the mean response obtained during the treatment period. Statistical significance was determined as \( P < 0.05 \), using the one tailed t-test.

The effect of four different doses of theophylline was studied on
MEPP amplitude and frequency. The dose schedule was randomized in each muscle allowing a one hour rinse with normal Ringer solution between doses. In each experiment the mean responses from control and treatment periods were calculated. The change in the treatment response was expressed as percent increase of the control value; i.e. 

\[(T-C/C \times 100)\]. The mean percent increase for each dose was plotted against the log of the dose. Frequency and amplitude distribution histograms were constructed for the optimal dose. On the histogram the abscissa represents MEPP frequency or amplitude; the ordinate, the percent incidence of each frequency or amplitude in the total sampling.

In another series of experiments, MEPP amplitude, rise-time, and 1/2 fall were investigated. MEPPS were displayed on the oscilloscope at a .2 msec/cm sweep speed. The MEPPS were recorded on single frames by randomly opening and closing the camera shutter. An average of twenty sequential frames were sampled for each control and treatment period using the same experimental protocol as described previously.

In experiments with dTc-depressed MEPPS, control measurements were made for ten minutes prior to the introduction of the blocking drug. d-Tubocurarine, 0.25 µg/ml, was perfused for an additional ten minutes and sample recordings taken during the last one minute. At this time, maximal depression of MEPP amplitude occurred.
Theophylline was added to the dTc perfusion media for ten additional minutes. Data was again collected during the last one minute of the experimental period.

Indirectly Stimulated Preparations

To study the effects of theophylline on EPPS, it was necessary to block muscle contraction. Two methods were utilized: 1) perfusion with d-tubocurarine (1.5-4.0μg/ml) or 2) transversally cutting the muscle fiber (Barstad and Lillihei, 1968). This latter method was described in the tenuissimus by Blaber (1970).

The criteria used in the selection of EPPS were the same as described for MEPPS. EPPS were recorded at a stimulation frequency of 0.5/sec. In this case, a calibration pulse was recorded before each evoked EPP on a triggered sweep of the oscilloscope. EPP amplitude, rise-time, 1/2 fall and resting potential were measured and analyzed in an identical manner in both types of preparations.

Trains of 100 EPPS were analyzed at a frequency of 50/sec for a duration of 2 sec in dTc-blocked preparations. The trains were applied at one minute intervals during the control and treatment periods. The first 15 EPPS of the train were designated the "head"; the remaining 85 EPPS were termed the "tail". The "tail" EPPS were further divided into 8 groups of 10 EPPS each. The amplitude of each EPP was measured and along with resting membrane potential and equilibrium
potential entered into a PDP Lab 8E digital computer.

The equilibrium potential \( E_{\text{EPP}} \) was assumed to be \(-15 \text{ mV} \) (Takeuchi and Takeuchi, 1959). Using the computer program the following parameters could be estimated: quantal content of the first EPP \( M_0 \), readily available stores \( N \), fraction of the available store released by the first EPP \( p \), quantum size \( q \), and mobilization of transmitter \( d_m \) (Elmqvist and Quastel, 1965b) (see Calculations).

Identical experimental procedures were performed using the transversally cut muscle fiber preparation. However, it was necessary to raise the stimulation frequency to 200/sec for 0.5 sec in order to estimate readily available stores (Blaber, 1970).

**Iontophoretic Application of Acetylcholine**

The method employed for iontophoretic application of ACh was similar to that described by Nastuk (1953). Pipettes with a resistance of 40–80 MΩ were used for acetylcholine ejection. In these pipettes, distilled water was replaced with 2.5 M acetylcholine Cl, instead of 3 M KCl. In solution, acetylcholine is in a positively charged state. By passing a positive electric field, the ACh will be ejected from the tip of the micropipette. To prevent the outward diffusion of ACh in between pulses, it is necessary to maintain an inward negative braking current. Therefore both channels of the Grass S88 stimulator were required, utilizing two SIU 5 Stimulus Isolation Units in series.
The ACh electrode mounted on a second Narishige micromanipulator was placed in series with a 47 kΩ resistor and a 20 MΩ shunt resistor. The ejection current was monitored as the voltage drop across the 47 kΩ resistor using a Grass P-18 DC amplifier (Figure 5).

The recording electrode was located at the endplate region of the muscle where MEPPS could be recorded. The second ACh containing electrode was situated, extracellularly, in close proximity to the endplate. Usually several repositioning attempts were needed to illicit an ACh potential of shortest possible duration. Ejection currents of 5-50 msec duration and approximately $2 \times 10^{-7}$ amps were used at a frequency of 0.05/sec. The braking current needed to maintain stable resting membrane potentials was $2 \times 10^{-9}$ amps. In these experiments the same experimental protocol was employed as described for the EPP and MEPP measurements.

**Moving Electrode Technique**

The experimental arrangement employing the sartorius muscle of the frog, Rana Pipiens, was similar to that described by Fatt (1950). Extreme care had to be taken in dissection as damage to only a few fibers resulted in a negativity appearing at the injured region in the electrical recording. The muscle was vertically mounted on a plexiglas holder with the pelvic end at the top and placed in a 50 ml glass chamber filled with frog Ringer solution. One agar cotton wick
Figure 5. Diagram of the circuit employed for iontophoretic application of ACh. Abbreviations are described in the text.
recording electrode was situated on the pelvic end, the other in the solution below the tibial end.

After soaking the muscle in ACh, 50–60 μg/ml, for two minutes, the solution was drained away at a rate of 50 ml/min. The potentials were recorded in reference to the level of the fluid which acted as a moving electrode. The magnitude of the ACh depolarization was measured through a Grass P-18 DC amplifier connected to a Harvard Apparatus recorder. To obtain consistent ACh responses a ten minute interval between applications was necessary. Theophylline was added only after two responses of equal magnitude were obtained. With carbachol, 10–15 μg/ml, the same procedure was followed, except thirty minute intervals were used between administrations.

Bath Perfusion of ACh

In this case, the intracellular microelectrode was placed at the end plate region of the cat tenuissimus muscle fiber. The usual recording system was used (Figure 4) with a Harvard Apparatus recorder connected to the DC amplifier. ACh, in a concentration of 10 μg/ml, was perfused through the muscle bath for 2–3 minutes with a ten minute interval between applications. When reproducible ACh-induced depolarizations had been attained, theophylline was added to the perfusion media. At the end of the ten minute treatment period, ACh was added to the theophylline-containing Ringer solution. The
magnitude of the ACh-induced depolarization in each experiment was compared before and after treatment with theophylline. An identical protocol was followed when carbachol, 1 µg/ml, was administered.

**Passive Membrane Characteristics**

In these experiments, a current was passed through the muscle membrane. The electrical arrangement of measuring the current passed and the electrotonic potential elicited by the muscle fiber was similar to that described by Fatt and Katz (1951) in frog sartorius and Boyd and Martin (1959) in cat tenuissimus.

The current passing microelectrode filled with 3M KCl was mounted on a second micromanipulator. A 47 KΩ resistor placed in series with the electrode served in the current monitoring system. To prevent shunting of the current through the solution a 50 MΩ resistor was also positioned in series with the electrode. The distance between the two electrodes and cell diameters were measured with a micrometer on the microscope at 86 X where 1 division = 12µ (Figure 6).

The recording electrode was inserted at the endplate region of the muscle fiber. The same cell was impaled with a second current passing electrode within 100µ of the recording electrode. This insertion caused the resting membrane potential to drop 5 to 10 mV. A shield to ground was placed between the two electrodes to reduce the
Figure 6. Diagram of the circuit employed in studies using two intracellular microelectrodes. Abbreviations are described in text.
stimulus artifact. Hyperpolarizing currents of 60 msec duration at 3 to 8 different intensities were passed through the muscle fiber. As the electrotonic potentials exhibited little variability, only three values were averaged for each current intensity. Currents of similar intensity were injected in the fiber ten minutes after treatment with theophylline. The amplitude of the electrotonic potential (mV) was plotted against the amount of current passed through the membrane. The resistance of the membrane (R) was determined as the slope of the plotted regression line. A paired statistical analysis was performed to compare the control and treatment values. The time constant, $T_m$, the membrane resistance of a unit area, $R_m$, and conductance, $G_m$, were also calculated from these data (see Calculations).

Depolarizing pulses of low intensity were also passed through the membrane during control and treatment periods. The current-voltage relationship is not linear with depolarizing currents of greater than one third the electrical threshold of the muscle membrane (Katz, 1948); therefore only hyperpolarizing currents were used for analysis.

**Equilibrium Potential of the EPP**

Using the constant current and extrapolation technique of Takeuchi and Takeuchi (1959, 1960a), the equilibrium potential of the EPP was determined. In these experiments, muscle action potentials
were blocked by transversally cutting the muscle fiber. The electrical arrangement and procedure were the same as that described for measuring membrane resistance (Figure 6).

Resting membrane potentials were altered by passing either hyperpolarizing or depolarizing currents through the cell membrane. The amplitude of the EPP was determined at three to seven different membrane potentials. At least five EPPs were averaged at each membrane potential. A plot of EPP size versus membrane potential was drawn for control and theophylline treatment values from each cell. Using a linear regression computer program, the X intercept was determined. This point, the membrane potential at which the EPP amplitude is zero, is termed the equilibrium potential of the EPP (E_{EPP}). The E_{EPP} could be determined directly only in the treatment period of the experiment, because the depolarizing current needed to reach the E_{EPP} resulted in permanent damage to the cell membrane.

The input membrane resistance, R, resistance for a unit area, R_m, and conductance, G_m, were also calculated as described in normal muscle fibers from these data (see Calculations).

Calculations

Quantal Analysis:

The computer program employed Martin's (1955) correction factor
for non-linear summation, since in a normal size EPP, the quanta do not summate in a linear fashion. The increment of potential contributed by each quantum to the total EPP becomes less and less as the number of units increases. By correcting the EPPS for non-linear summation using Martin's Correction Factor (1955), the amplitudes of the EPPS conform to a Poisson distribution. The EPPS in dTc-blocked preparations and in transversally cut muscle fiber preparations are corrected as follows:

$$EPP' = \frac{EPP}{(RP - E_{EPP} - EPP)} \times (RP - E_{EPP})$$  \hspace{1cm} (1)

where RP = the resting membrane potential and $E_{EPP}$ = equilibrium potential of the EPP. The corrected EPP amplitudes in the tail follow a Poisson distribution (Martin, 1955). By applying Poisson theory, the variance of each of the 8 blocks of 10 EPPS in the tail is calculated:

$$\text{Variance}_{EPP} = \frac{\overline{EPP}}{SD^2}$$  \hspace{1cm} (2)

where $\overline{EPP}$ = the mean amplitude of the EPP in each block and SD = the standard deviation. From equation 2, the coefficient of variation (CV) is calculated:

$$CV = \frac{SD}{\overline{EPP}}$$  \hspace{1cm} (3)

Del Castillo and Katz (1954a) defined the quantal content (m) of the EPP as:

$$m^2 = \frac{1}{CV^2}$$  \hspace{1cm} (4)
Substituting equation 3 in equation 4:

\[ m = \frac{EPP^2}{\text{variance}_{EPP}} \]  

(5)

Based on the premise of the quantal theory of transmitter release (del Castillo and Katz, 1954a), quantal content is also defined as:

\[ m = \frac{EPP}{\text{MEPP}} \]  

(6)

Assuming that the variation in the size of the quantum relative to the mean quantum size (\( \bar{q} \)) of the MEPP is negligible (Edwards and Ikeda, 1962),

\[ \bar{q} = \frac{\text{MEPP}}{\text{EPP}} \]  

(7)

Combining equations 6 and 7:

\[ \bar{q} = \frac{\text{EPP}}{m} \]  

(8)

and substituting equation 5 in equation 7:

\[ q = \frac{\text{variance}_{EPP}}{\text{EPP}} \]  

The mean quantum size is estimated from each block in the tail and used to calculate the quantal content of the EPPS in the "head" of the train. The mean quantal content of the 80 EPPS in the "tail" is divided by the pulse interval to give an estimate of the rate of mobilization of transmitter (\( d_m \)). The quantal content of the first 15 EPPS versus the cumulative number of quanta released with each impulse is plotted and the correlation coefficient is computed for each point. From the linear regression plot, the intercepts on the X and Y axis are also determined. The Y-intercept represents the quantal content of the first EPP, \( (M_o) \) and the X-intercept is an estimate of
the store of transmitter immediately available for release (N) (Elmqvist and Quastel, 1965b). Therefore, the fraction of the readily available store released by the first impulse is calculated as:

\[ M_0 = N \cdot p \]  

(del Castillo and Katz, 1954a)  

(9)

where \( N \) = readily available store estimated from the X-intercept of the fitted regression line using the first 5 points.

**Passive Membrane Characteristics:**

The basic equation determining membrane resistance (R) is given by Ohm's Law:

\[ R = \frac{V}{I} \]  

(1)

where \( V \) = voltage of the electrotonic potential and \( I \) = amount of current passed through the membrane, calculated from the voltage drop across a 47K\( \Omega \) resistor. Current is plotted against voltage at each point and the resistance is computed as the slope of the regression line fitted through these points. A paired comparison is performed for each cell using the slope of the line before and after drug treatment.

Further calculations made use of the cable equation of Hodgkin and Rushton (1946) describing the potential change (V) produced by a constant current (I) through the membrane:

\[ V = \frac{1}{2} I \sqrt{r_m} \cdot r_i \exp \left( -\frac{X}{\sqrt{r_m} / r_i} \right) \]  

(2)

where \( X \) = the electrode separation (\( \mu \)), \( r_m \) = the transverse resistance
of a unit length of fiber (Ω/cm), and \( r_1 \) = the internal myoplasm resistance per unit length (Ω/cm). From equation 1, with \( X = 0 \) (or within 50 \( \mu \)),

\[
R = \frac{1}{2} \sqrt{\frac{r_m}{r_1}}
\]

(3)

According to Katz and Thesleff (1957),

\[
R_m = \pi \cdot d \cdot r_m
\]

(4)

where \( R_m \) = the resistance for a unit area, and \( d \) = diameter of the muscle fiber and,

\[
R_i = \pi \cdot (d/2)^2 \cdot r_1
\]

(5)

where \( R_i \) = the internal resistance of the myoplasm. Combining equations 3, 4, and 5,

\[
R_m = \left( R \cdot \pi \right)^2 \cdot \frac{d^3}{R_i}
\]

(6)

\( R_i \) is assumed to be 125Ωcm at 37°C as described by Boyd and Martin (1959) in cat tenuissimus muscle. \( T_m \), the time constant of the cable, is the time for the electrotonic potential to fall to 83% of its maximum steady value with the two electrodes separated by less than 50 \( \mu \) (Hodgkin and Rushton, 1946). The conductance of a unit area of membrane, \( G_m \), is the inverse of the resistance (\( R_m \)):

\[
G_m = \frac{1}{R_m}
\]

To evaluate the conductance change produced by MEPPS, ACh depolarizations, and carbachol depolarizations, the equation of Katz and Thesleff (1957) is used:
\[ V = (RP - E_{EPP}) \times G \times R \quad (1) \]

therefore,

\[ G = \frac{V}{(RP - E_{EPP}) \times R} \quad (2) \]

where \( V \) = the amplitude (mV) obtained experimentally, \( RP \) = resting membrane potential observed during MEPP recording (Table 2), and \( R \) = membrane resistance as measured in passive membrane determinations (Table II). \( E_{EPP} \) = equilibrium potential of the EPP, -15 mV (Takeuchi and Takeuchi, 1959, 1960a). Since the \( E_{EPP} \) shifted 6 mV with theophylline in the cut fiber preparations, a value of -9 mV is used for treatment calculations. \( G \) = conductance change produced by MEPPs and ACh and carbachol depolarizations, as computed from equation 2.

**Solutions and Drugs**

The muscle was bathed in Krebs-Henseleit Solution (1932) which consisted of:

- \( \text{NaCl} = 115 \text{ mM} \)
- \( \text{KCl} = 4.6 \text{ mM} \)
- \( \text{KH}_2\text{PO}_4 = 1.15 \text{ mM} \)
- \( \text{NaHCO}_3 = 24.1 \text{ mM} \)
- \( \text{CaCl}_2 = 2.46 \text{ mM} \)
- \( \text{MgSO}_4 = 1.15 \text{ mM} \)
- \( \text{Glucose} = 8.85 \text{ mM} \)
Theophylline, as the ethylenediamine salt, *(Aminophylline®*, Searle), dissolved in this solution was prepared fresh each day. For every molecule of Aminophylline there are two molecules of theophylline and one molecule of ethylenediamine. Since ethylenediamine was present in all investigations performed, it was necessary to carry out control experiments using this compound. The effect of ethylenediamine was studied on the frequency and amplitude of MEPPS at the concentration present in the optimal dose of 0.9 mM Aminophylline containing 1.8 mM theophylline and 0.9 mM ethylenediamine.

Other drugs used in this study were d-Tubocurarine Cl (Abbott), acetylcholine Cl (Sigma) and carbachol (Carcholin®, Merck, Sharp, & Dohme).

The set of experiments using frog muscle employed Frog Ringer solution which was composed of:

\[
\begin{align*}
\text{NaCl} & = 112 \text{ mM} \\
\text{KCl} & = 2 \text{ mM} \\
\text{CaCl}_2 & = 1.8 \text{ mM} \\
\text{NaHCO}_3 & = 1.2 \text{ mM}
\end{align*}
\]

The pH of the control Ringer solution was 7.4; the addition of 0.9 mM aminophylline produced a pH of 7.6. A change in pH of this magnitude and direction could not account for the experimental effects observed during theophylline treatment *(Hubbard, et al., 1968a)*.
CHAPTER III

RESULTS
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RESULTS

Effect of Theophylline on MEPPS

Pre- and postsynaptic effects of theophylline treatment were initially explored on MEPP frequency and amplitude.

The effects of 0.45, 0.9, 1.8, and 3.6 mM theophylline on MEPP frequency are shown in Figure 7. The logarithm of the mM dose is plotted on the abscissa and the percentage increase in MEPP frequency compared to controls, on the ordinate. Each point represents a mean of four experiments in muscle obtained from four cats. As can be seen, the two lower doses produced a 50% increase in MEPP frequency. At the 1.8 mM level, MEPP frequency was maximally increased (200%). The highest dose tested (3.6 mM theophylline) produced a marked depolarization of the resting membrane potential, decreasing the amplitude of MEPPS below noise level. As a result, there was probably a significant number of MEPPS which could not be measured. It was difficult, in general, to maintain the electrode in the cell after 3.6 mM theophylline treatment since some degree of contracture usually dislodged the recording electrode.

The effect of theophylline on MEPP amplitude is described in Figure 8. The log mM dose of theophylline is represented on the
Figure 7. Effect of 0.45, 0.9, 1.8, and 3.6 mM theophylline on MEPP frequency. Abscissa = log concentration (mM); ordinate = percent increase of control frequency. Points are plotted as mean ± standard error. Each mean value was pooled from four experiments at each dose level.
Figure 8. Effect of 0.45, 0.9, 1.8, and 3.6 mM theophylline on MEPP amplitude. Abscissa = log concentration (mM); ordinate = percent increase of control amplitude. Points are plotted as mean ± standard error. Each mean value was pooled from four experiments at each dose level.
abscissa, and in this case, the percentage increase in MEPP amplitude is plotted on the ordinate. A dose dependent relationship resulted at 0.45, 0.9, and 1.8 mM, as MEPP amplitude increased with increasing doses of theophylline. Again at the 3.6 mM dose, a decrease in MEPP amplitude occurred due to the large depolarization of the resting membrane potential.

From these graphs (Figures 7 & 8), it can be seen that the maximal effect of theophylline on MEPP amplitude and frequency occurred at a concentration of about 1.8 mM. Based on these results, the 1.8 mM dose of theophylline was used in all subsequent experiments.

To illustrate the specific nature of the mean increases in amplitude and frequency produced by theophylline, distribution histograms were compiled for the 1.8 mM dose from four experiments (four cats). The distribution for MEPP frequency is shown in Figure 9. A typical skewed Poisson type of distribution (Fatt and Katz, 1952) is depicted in the control graph (solid line) with a mean of 2 MEPPS/sec. Treatment with 1.8 mM theophylline (dashed line) resulted in a shift of the mean distribution to the right, increasing the frequency to 5 MEPPS/sec.

The distribution of MEPP amplitudes is shown in Figure 10. The shift of the control amplitude distribution to the larger amplitudes after treatment with 1.8 mM theophylline is evident. This is reflected in the change in mean amplitude from 0.62 mV (N=244) in control to 0.77 mV (N=629) when treated with 1.8 mM theophylline. An
Figure 9. The frequency distribution of control MEPPS (solid line) and MEPPS after treatment with 1.8 mM theophylline (dashed line). The mean frequency in control and treated distributions is 2 MEPPS/sec and 5 MEPPS/sec, respectively.
Figure 10. The amplitude distribution of control MEPPS (solid line) and MEPPS after treatment with 1.8 mM theophylline (dashed line). The mean amplitude in control and treated distributions is 0.62 mV (N=244) and 0.77 mV (N=629), respectively.
example of the MEPP recordings used in Figures 9 & 10 is shown in Figure 11. The increased frequency and amplitude of the MEPPs seen with 1.8 mM theophylline is apparent in the right panel.

Since the 1.8 mM dose of theophylline also contained 0.9 mM ethylenediamine additional control experiments were performed. Table 1 summarizes the effect of 0.9 mM ethylenediamine on the frequency and amplitude of the MEPP. Analysis demonstrated that ETDA caused a slight but statistically significant increase in MEPP frequency but had no effect on MEPP amplitude. Resting membrane potentials were unaffected. The degree of frequency increase seen with 0.9 mM ethylenediamine (i.e. that concentration present in the aminophylline mixture) was less than 10% the increase seen with 1.8 mM theophylline.

To investigate the effect of 1.8 mM theophylline on MEPP time course in detail, MEPP amplitude, rise-time and 1/2 fall were studied. Table 2 summarizes the effect of theophylline on these parameters. MEPP amplitude, rise-time and 1/2 fall were all significantly increased by 1.8 mM theophylline. In addition, there was a small but statistically significant depolarization of the resting membrane potential. These effects of theophylline were reversible when perfusion with Ringer solution was resumed.

These data suggest that theophylline possesses a presynaptic site of action as well as a postsynaptic site, i.e. both MEPP frequency and amplitude increase. Further delineation of the
Figure 11. Effect of 1.8 mM theophylline on MEPP frequency and amplitude. Calibration signal (single dot) is evident in tracings 1, 4, 5, and 7, in the left panel, and in tracings 2, 3, 5, and 6, in the right panel. Each tracing is 1 sec duration. Calibration = 0.5 mV.
TABLE 1

EFFECTS OF 0.9 mM ETHYLENEDIAMINE ON
MEPP FREQUENCY AND AMPLITUDE

<table>
<thead>
<tr>
<th></th>
<th>Frequency (MEPPS/sec)</th>
<th>Amplitude (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.69 ± 0.55</td>
<td>0.63 ± 0.01</td>
</tr>
<tr>
<td>Treated</td>
<td>2.07 ± 0.58*</td>
<td>0.59 ± 0.01</td>
</tr>
</tbody>
</table>

Percent Difference %  
+27.62 ± 7.88   
-7.29 ± 5.14

Data expressed as mean ± standard error

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test.
TABLE 2

EFFECT OF 1.8 mM THEOPHYLLINE ON MEPP AMPLITUDE AND TIME COURSE

<table>
<thead>
<tr>
<th></th>
<th>R.P. (mV ± S.E.)</th>
<th>Amplitude (mV ± S.E.)</th>
<th>Rise-time (msec ± S.E.)</th>
<th>1/2 fall (msec ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82.0 ± 4.6</td>
<td>.82 ± .03</td>
<td>0.58 ± .01</td>
<td>.92 ± .01</td>
</tr>
<tr>
<td>(N=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>77.7 ± 4.8*</td>
<td>1.07 ± .03*</td>
<td>0.72 ± .01*</td>
<td>1.19 ± .01*</td>
</tr>
<tr>
<td>(N=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant difference (p < 0.05) paired control and treated values, one tailed t-test.

R.P. = Resting Potential
facilitatory sites of action of theophylline involved an investigation of evoked transmitter release.

**Effect of Theophylline on EPP Amplitude and Time Course**

The effects of theophylline on EPP amplitude and time course in dTc-blocked muscles (6 cats) are summarized in Table 3. Theophylline significantly increased EPP amplitude, rise-time and 1/2 fall. The anti-dTc action was sufficient in some cases to increase EPP amplitude to threshold levels, since it was sometimes observed that fibers began twitching after 5-10 minutes of perfusion with this dose of theophylline. A small but statistically significant depolarization of the resting membrane potential occurred. Theophylline's effects on resting potential may be independent of the effects on EPPs, as evidenced by the fact that the resting potentials were restored to pretreatment levels prior to restoration of control EPP amplitudes. A typical EPP response to theophylline after 10 minutes of exposure to the drug is shown in Figure 12. Note that although the time course of the EPP was significantly prolonged by theophylline (Table 3), it is not evident in the dTc-blocked preparation (Figure 12).

The actions of theophylline on five cut fiber preparations are analyzed in Table 4. In this case, theophylline also increased the amplitude of EPPs; this effect was not statistically significant unless corrections were made for non-linear summation. Corrections of the
TABLE 3

EFFECT OF 1.8 mM THEOPHYLLINE ON 
d-TUBOCURARINE BLOCKED EPPS

<table>
<thead>
<tr>
<th></th>
<th>R.P. (mV ± S.E.)</th>
<th>Amplitude (mV ± S.E.)</th>
<th>Rise-time (msec ± S.E.)</th>
<th>1/2 fall (msec ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N=6)</td>
<td>74.7 ± 1.9</td>
<td>1.94 ± 0.04</td>
<td>0.43 ± 0.01</td>
<td>0.66 ± 0.01</td>
</tr>
<tr>
<td>Treated (N=6)</td>
<td>70.0 ± 2.3*</td>
<td>3.20 ± 0.06*</td>
<td>0.54 ± 0.02*</td>
<td>0.75 ± 0.01*</td>
</tr>
</tbody>
</table>

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test.

R.P. = Resting Potential
Figure 12. EPPS recorded in 3 μg/ml dTc-blocked muscle. A, control; B, 1.8 mM theophylline. Calibration = 1 mV x 1 msec; stimulation frequency = 0.5/sec.
### TABLE 4

**EFFECT OF 1.8 mM THEOPHYLLINE ON EPPS IN CUT FIBER PREPARATIONS**

<table>
<thead>
<tr>
<th></th>
<th>R.P. (mV ± S.E.)</th>
<th>Amplitude (mV ± S.E.)</th>
<th>Rise-time (msec ± S.E.)</th>
<th>1/2 fall (msec ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>44.0 ± 3.5</td>
<td>9.77 ± 0.11</td>
<td>0.53 ± .02</td>
<td>0.96 ± .02</td>
</tr>
<tr>
<td>(N=5)</td>
<td></td>
<td>(15.44 ± 2.12)+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Treated</strong></td>
<td>40.8 ± 3.5</td>
<td>10.91 ± 0.20</td>
<td>0.71 ± .03*</td>
<td>1.31 ± .02*</td>
</tr>
<tr>
<td>(N=5)</td>
<td></td>
<td>(19.58 ± 2.65)+*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test

R.P. = Resting Potential

+ Corrected for non-linear summation
EPP amplitude are necessary in the depolarized condition characteristic of the cut muscle fiber preparation (Barstad and Lillihei, 1968), in order to accurately estimate quantal content by the coefficient of variation method (Martin, 1955). The depolarization due to theophylline added to the depolarization of the transversally cut muscle fiber, therefore the correction for non-linear summation had a marked effect on the estimates of EPP amplitude. It should be noted that if the theophylline effect was confined to membrane depolarization, a small EPP would result, as in this situation the resting membrane potential is shifted closer to the equilibrium potential; this however was not the case. The extent of the prolongation of rise-time and 1/2 fall were remarkably similar to those recorded in MEPP experiments (c.f. Tables 2 & 4). A typical recording of the effects of theophylline on EPPS in cut muscle fiber preparation is shown in Figure 13. Notice that the prolonged 1/2 fall during theophylline treatment is more obvious in the cut muscle fiber preparation than in the dTc-blocked preparation (Figure 12).

It was observed that the rise-time and 1/2 fall of MEPPS and EPPS were significantly increased during theophylline treatment (Tables 2, 3, & 4). The increase in the MEPP or EPP time course could be associated with a presynaptic event. To investigate this possibility the effects of theophylline on trains of EPPS were studied in the dTc-blocked muscle and the cut muscle fiber.
Figure 13. EPPS recorded in cut fiber muscle. A, control; B, 1.8 mM theophylline. Calibration = 5 mV x 1 msec; stimulation frequency = 0.5/sec.
**Effect on Trains of EPPS**

Figure 14 demonstrates the effects of theophylline on trains of EPPS in dTc-blocked muscle. The control recordings are indicated in the two upper panels (A) and the response to 1.8 mM theophylline in the bottom two panels (B). The first seven and the last seven EPPS of the train of 100 EPPS are shown in the left and right panels, respectively. At the "head" of the trains, a rapid decline in the amplitude of each succeeding EPP is observed; this is followed by a gradual decline. It is evident in the bottom two panels (B) that theophylline treatment increased the amplitude of all EPPS in the train.

An example of a train of EPPS obtained in the cut fiber preparation is shown in Figure 15. In the case of the cut fiber, the control EPPS do not decline as rapidly as EPPS obtained in dTc-blocked fibers (cf. Figures 14 & 15). Theophylline treatment only slightly increased the amplitude of the first EPP (cf. A & B, Figure 15). The first EPP amplitude and the 95th EPP amplitude in the tail in seven experiments using six cats in dTc-blocked muscle and seven experiments using six cats in cut muscle fiber preparations are compared in Table 5. In the case of dTc-treated muscle, the amplitude of the 1st and 95th EPP are significantly larger after theophylline treatment. In contrast, in the cut muscle fiber, the increase in amplitude of the first EPP is not statistically significant from controls, and the amplitude of the 95th EPP is significantly decreased.
Figure 14. Trains of EPPS recorded in 3 µg/ml d-tubocurarine blocked muscle. A, first 7 EPPS in the left panel and last 7 EPPS in the right panel in a train of 100 in the control period; B, first 7 EPPS in the left panel and last 7 EPPS in the right panel in a train of 100, ten minutes after the addition of 1.8 mM theophylline. Calibration = 1 mV; stimulation frequency = 50/sec.
Figure 15. Trains of EPPS in cut muscle fiber. 
A, first 20 EPPS in the left panel and last 20 EPPS in the right panel in a train of 100 in the control period; B, first 20 EPPS in the left panel and last 20 EPPS in the right panel in a train of 100, ten minutes after the addition of 1.8 mM theophylline. Calibration = 5 mV; stimulation frequency = 200/sec.
**TABLE 5**

**EFFECT OF 1.8 mM THEOPHYLLINE ON EPP AMPLITUDES IN TRAINS**

<table>
<thead>
<tr>
<th></th>
<th>Mean Amplitude of the First EPP (mV ± S.E.)</th>
<th>Mean Amplitude of the 95th EPP (mV ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>dTc Blocked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preparations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncorrected EPP</td>
<td>$2.54 \pm 0.35$</td>
<td>$3.29 \pm 0.36^*$</td>
</tr>
<tr>
<td>Corrected EPP+</td>
<td>$2.67 \pm 0.39$</td>
<td>$3.51 \pm 0.41^*$</td>
</tr>
<tr>
<td>Cut Fiber</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preparations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncorrected EPP</td>
<td>$9.54 \pm 1.12$</td>
<td>$10.03 \pm 1.38$</td>
</tr>
<tr>
<td>Corrected EPP+</td>
<td>$16.78 \pm 4.45$</td>
<td>$24.19 \pm 7.45$</td>
</tr>
</tbody>
</table>

*+Corrected for non-linear summation

*Statistically significant difference ($p < 0.05$) between paired control and treated values, one tailed t-test
Components of transmitter release as described by Elmqvist and Quastel (1965b) were also investigated using trains of EPPS. The effects of 1.8 mM theophylline were analyzed in both dTc-paralyzed muscle and in the cut fiber preparation.

The results obtained in dTc-blocked muscle are summarized in Table 6. Analysis demonstrated that although the amplitude of the first EPP increased significantly in dTc-treated muscle, the quantal content, mobilization, available stores, and probability of release were not affected by theophylline treatment. Only quantum size showed a significant increase relative to control. Resting potential declined slightly.

In the cut fiber preparation (Table 7), however, presynaptic effects were uncovered. Theophylline increased the quantal content and probability of release while it decreased readily available stores. Mobilization was unaffected, as was quantum size. The resting membrane potential declined about 4 mV.

The fact that theophylline increased EPP amplitude in dTc-blocked muscle by increasing quantum size without affecting quantal content, and prolonged the rise-time and 1/2 fall in the presence and absence of d-tubocurarine, suggested that theophylline may possess post-synaptic actions. The observation that theophylline increased the amplitude, rise-time and 1/2 fall of MEPPS further supported this evidence. The question then arose whether theophylline could
TABLE 6

EFFECT OF 1.8 mM THEOPHYLLINE ON NEUROMUSCULAR TRANSMISSION IN dTc BLOCKED PREPARATIONS

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantum Size (q)</td>
<td>.007 ± .001</td>
<td>.010 ± .002*</td>
</tr>
<tr>
<td>Quantal Content (Mo)</td>
<td>396 ± 64</td>
<td>389 ± 69</td>
</tr>
<tr>
<td>Mobilization (q/msec)</td>
<td>7.81 ± 1.20</td>
<td>7.26 ± 1.05</td>
</tr>
<tr>
<td>Available Stores (N)</td>
<td>2045 ± 305</td>
<td>1991 ± 273</td>
</tr>
<tr>
<td>Probability of Release (p)</td>
<td>.199 ± .015</td>
<td>.195 ± .017</td>
</tr>
<tr>
<td>Resting Potential (R.P.)</td>
<td>75.3 ± 4.1</td>
<td>73.0 ± 4.6</td>
</tr>
</tbody>
</table>

N=7; stimulation frequency = 50/sec.

*Statistically significant difference (p<0.05) between paired control and treated values, one tailed t-test.
TABLE 7

EFFECT OF 1.8 mM THEOPHYLLINE ON NEUROMUSCULAR TRANSMISSION IN CUT FIBER PREPARATIONS

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantum Size (q)</td>
<td>0.070 ± 0.014</td>
<td>0.068 ± 0.013</td>
</tr>
<tr>
<td>Quantal Content (Mo)</td>
<td>238 ± 22</td>
<td>340 ± 35*</td>
</tr>
<tr>
<td>Mobilization (q/msec)</td>
<td>20.92 ± 1.73</td>
<td>21.37 ± 1.46</td>
</tr>
<tr>
<td>Available Stores (N)</td>
<td>4025 ± 439</td>
<td>3237 ± 307*</td>
</tr>
<tr>
<td>Probability of Release (p)</td>
<td>0.062 ± 0.007</td>
<td>0.112 ± 0.017*</td>
</tr>
<tr>
<td>Resting Potential (R.P.)</td>
<td>43.7 ± 1.92</td>
<td>39.4 ± 2.95</td>
</tr>
</tbody>
</table>

N=7; stimulation frequency = 200/sec.

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test.
reverse the postsynaptic action of dTc on MEPPS.

Effect on dTc-Depressed MEPPS

The effects of theophylline on MEPP amplitudes were examined in the presence of a concentration of d-tubocurarine sufficient to depress MEPP amplitudes. A series of six experiments in three cats is summarized in Table 8. Perfusion with 0.25 μg/ml dTc significantly depressed MEPP amplitude. When 1.8 mM theophylline was added to the perfusate, the MEPPs progressively increased in amplitude over a 10 minute treatment period until essentially control amplitudes were obtained. A typical experiment illustrating this phenomenon is shown in Figure 16. These results indicate that theophylline possessed a postsynaptic anti-dTc action. To provide definitive evidence, it was necessary to measure the effect of theophylline on exogenously applied acetylcholine.

Effect of Theophylline on Iontophoretically Applied ACh Potentials

Figure 17 demonstrates a facilitatory effect of theophylline on ACh potentials. As can be seen, theophylline produced an increase in the amplitude and time course of the ACh potential that was reversible. However, this facilitation and reversibility of the ACh potential amplitude was only evident in 2 of 20 experiments. In experiments in which MEPPs could be measured, it was noted that
TABLE 8

EFFECT OF 1.8 mM THEOPHYLLINE ON
dTc DEPRESSED MEPPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>R.P. (mV ± S.E.)</th>
<th>MEPP Amplitude (mV ± S.E.)</th>
<th>Statistical Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Control (N=6)</td>
<td>72.8 ± 1.0</td>
<td>0.61 ± .01</td>
<td>A vs B*</td>
</tr>
<tr>
<td>(B) 0.25 μg/ml dTc (N=6)</td>
<td>72.7 ± 1.5</td>
<td>0.47 ± .01</td>
<td>B vs C*</td>
</tr>
<tr>
<td>(C) 0.25 μg/ml dTc + 1.8 mM Theophylline (N=6)</td>
<td>65.7 ± 3.3</td>
<td>0.57 ± .01</td>
<td>A vs C</td>
</tr>
</tbody>
</table>

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test.

R.P. = Resting Potential

†Statistical comparisons are between MEPP amplitudes.
Figure 16. MEPPS recorded in the presence and absence of d-tubocurarine. A, uppermost panels, are typical control recordings. B, middle panels, MEPPS recorded after 10 minutes perfusion with 0.25 μg/ml dTc. Calibration signal is evident in all panels. C, lower panels, recorded after 10 minutes perfusion with 0.25 μg/ml dTc plus 1.8 mM theophylline. Calibration evident in lower left panel. Calibration = 0.5 mV x 1 msec.
Figure 17. Effect of 1.8 mM theophylline on ACh potentials (upper traces). ACh ejection pulses (lower traces) = 5 msec duration and $7.5 \times 10^{-7}$ amps; calibration = 5 mV x 20 msec.
although the ACh potential did not change, MEPPS were increased. This observation is illustrated in Figure 18. It was suspected that theophylline may somehow induce a movement artifact in the ACh potential measurements. In an effort to eliminate the artifact, the moving electrode technique (Fatt, 1950) was employed to study the effect of theophylline on ACh-induced depolarizations in frog sartorius muscle (see Methods).

**Moving Electrode Technique**

Figure 19 depicts the effect of theophylline on ACh-induced depolarizations in frog sartorius muscle. The two points of greatest depolarization in the control (left hand tracing) represent those areas on the frog muscle with the highest density of endplates. Theophylline treatment (right tracing) produced a significant potentiation of the depolarization which was reversible. This type of facilitation coupled with prolongation of the time course of MEPPS and EPPS is characteristic of cholinesterase inhibition. To eliminate the possibility that theophylline may possess anticholinesterase properties, a stable choline ester, carbachol was used.

The effect of theophylline on carbachol-induced depolarizations is demonstrated in Figure 20. Although there is a slight potentiation of the carbachol depolarization, the effect did not prove statistically significant. Table 9 summarizes the data from five experiments using
Figure 18. Effect of 1.8 mM theophylline on ACh potentials and MEPPS (upper traces). ACh ejection pulses (lower traces) = 14 msec duration and $5.3 \times 10^{-7}$ amps; calibration = 5 mV x 20 msec.
Figure 19. Effect of 1.8 mM theophylline on the depolarization produced by 50 μg/ml ACh in frog sartorius muscle.
Figure 20. Effect of 1.8 mM theophylline on the depolarization produced by 15 μg/ml carbachol in frog sartorius muscle.
### TABLE 9

**EFFECT OF 1.8 mM THEOPHYLLINE ON ACh AND CARBACHOL DEPOLARIZATIONS IN FROG SARTORIUS MUSCLE**

<table>
<thead>
<tr>
<th></th>
<th>ACh (mV ± S.E.)</th>
<th>Carbachol (mV ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 5</td>
<td>N = 3</td>
</tr>
<tr>
<td>Control</td>
<td>1.07 ± .19</td>
<td>4.27 ± 1.23</td>
</tr>
<tr>
<td>Treated</td>
<td>2.36 ± .24*</td>
<td>5.07 ± 1.39</td>
</tr>
</tbody>
</table>

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test.
ACh and five experiments with carbachol.

Since all prior data had been obtained in cat tenuissimus muscle, we felt a valid extrapolation could not be drawn from the results on frog sartorius muscle. It was therefore necessary to investigate the effect of theophylline on exogenously applied ACh in cat muscle.

**Bath Perfusion of Acetylcholine**

Perfusion with ACh initiated depolarizations which were recorded at the endplate region of the muscle fiber using intracellular micro-electrodes. Theophylline produced a facilitation of the ACh-induced depolarization, as illustrated in Figure 21. On the other hand, when carbachol was perfused, theophylline had no significant potentiating action (Figure 22). The effects of theophylline on carbachol and ACh depolarizations are analyzed in Table 10. These experiments suggest that theophylline has a postsynaptic site of action and, based on the results obtained from carbachol perfusion, that this action may be attributed to an anticholinesterase effect. However, postsynaptic increases in amplitude and time course of MEPPS and EPPS may also be explained by an increase in the resistance of the muscle membrane. To investigate this possibility the passive membrane characteristics were studied.
Figure 21. Effect of 1.8 mM theophylline on the depolarization produced by 10 μg/ml ACh in cat tenuissimus muscle recorded at the endplate region with intracellular microelectrodes.
Figure 22. Effect of 1.8 mM theophylline on the depolarization produced by 1 μg/ml carbachol at the endplate region of the cat tenuissimus muscle with intracellular microelectrodes.
TABLE 10

EFFECT OF 1.8 mM THEOPHYLLINE ON ACh AND CARBACHOL DEPOLARIZATIONS IN CAT TENUISSIMUS MUSCLE

<table>
<thead>
<tr>
<th></th>
<th>ACh (mV ± S.E.)</th>
<th>Carbachol (mV ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 6</td>
<td>N = 6</td>
</tr>
<tr>
<td>Control</td>
<td>3.58 ± .49</td>
<td>7.70 ± .56</td>
</tr>
<tr>
<td>Treated</td>
<td>6.33 ± .44*</td>
<td>8.90 ± .87</td>
</tr>
</tbody>
</table>

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test.
Passive Membrane Characteristics

In these experiments, the endplate region of the muscle was impaled with one electrode, while a second electrode was inserted in the same fiber within an average distance of 46.5 microns. The insertion of the second electrode caused the resting membrane to decline from 70 mV to 60.9 mV. Treatment with theophylline resulted in a further depolarization of the resting membrane potential to 44.8 mV; after rinsing with normal Ringer solution, this latter depolarization returned to control values. Figure 23 illustrates a sample record taken from an experiment. The upper tracings are the evoked electrotonic potentials, the lower tracings are the current pulses. After ten minutes treatment with theophylline the electrotonic potentials decreased in magnitude, though the currents passed were equal to those of the control (c.f. right and left tracings). The small deflections in the electrotonic potential tracings are MEPPS.

A current-voltage plot of a typical experiment is illustrated in Figure 24. The slope of the regression line after theophylline treatment is decreased (open circles). This effect is reversible. The data from 8 experiments (7 cats) is analyzed in Table 11. The membrane resistance (\(R\)) is significantly decreased in the presence of theophylline. Resistance for a unit area (\(R_{m}\)) and membrane time constant (\(T_{m}\)) are similarly affected. Consequently, the membrane conductance (\(G_{m}\)) increased, indicating a change in permeability. All the
Figure 23. Electrotonic potentials (upper traces) produced by the application of 0.89, 1.06, and $1.31 \times 10^{-8}$ amp current pulses of 60 msec duration (lower traces) before and after treatment with 1.8 mM theophylline. Calibration = 5 mV x 5 msec.
Figure 24. Effect of 1.8 mM theophylline on membrane resistance. Control = solid circles; treatment with 1.8 mM theophylline = open circles; post-treatment control after 30 minutes rinsing with normal Ringer solution = crosses.
TABLE II

EFFECT OF 1.8 mM THEOPHYLLINE ON
PASSIVE MEMBRANE CHARACTERISTICS

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 8</td>
<td>N = 8</td>
</tr>
<tr>
<td>Input Resistance (R)</td>
<td>4.24 ± 0.37</td>
<td>3.13 ± 0.38*</td>
</tr>
<tr>
<td>(x10^5 ohms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time Constant (T_m)</td>
<td>5.4 ± 0.3</td>
<td>3.9 ± 0.3*</td>
</tr>
<tr>
<td>(msec)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance of a unit area (R_m)</td>
<td>6104 ± 1043</td>
<td>3404 ± 755*</td>
</tr>
<tr>
<td>(ohm cm^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conductance of a unit area (G_m)</td>
<td>1.95 ± 0.27</td>
<td>4.19 ± 0.84*</td>
</tr>
<tr>
<td>(x10^-4 mho/cm^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting Potential (R.P.)</td>
<td>61.8 ± 1.0</td>
<td>44.8 ± 1.9*</td>
</tr>
<tr>
<td>(mV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter (d)</td>
<td>75 ± 3</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>(μ)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test
correlation coefficients of these regression lines were in the range 0.9941 to 0.9997.

The control values of membrane resistance ($R_m$) and time constant ($T_m$) are in complete agreement with those reported by Boyd and Martin (1959) in cat tenuissimus muscle. However, the control $R_m$ value, $6104 \Omega \text{cm}^2$, does not agree with the value, $1430 \Omega \text{cm}^2$, reported by Boyd and Martin. This disparity may perhaps be explained by the differences in the cell diameter reported in these two studies. In the present study diameters, measured directly from the cells impaled in living preparations, averaged 75μ. In contrast Boyd and Martin's measurements obtained in fixed preparations estimated a cell diameter of 44μ.

An increase in the membrane resistance of a muscle fiber would be accompanied by an increased MEPP amplitude (Katz and Thesleff, 1957). Thus, the decrease in membrane resistance observed in these experiments would not account for the increased MEPP and EPP amplitude; however, it may be related to the resting membrane potential depolarization which occurred with theophylline treatment in all experiments.

Since the prior experiments did not explain the basis of the increased MEPP and EPP amplitude, it was decided to investigate the equilibrium potential of the EPP ($E_{EPP}$) as a shift of the $E_{EPP}$ to a more positive level could result in an increased MEPP and EPP amplitude (Takeuchi, 1963).
Equilibrium Potential of the Endplate Potential

The equilibrium potential of the EPP was determined in the transversally cut muscle fiber preparation of Barstad and Lillihei (1968) using the constant current and extrapolation technique of Takeuchi and Takeuchi (1959, 1960a). The cut muscle fiber preparation was utilized to analyze a theophylline action not evident in d-Tc-blocked muscle.

A plot of EPP amplitude at different membrane potentials from a typical experiment is shown in Figure 25. The equilibrium potential is determined by a linear regression as the point of zero EPP amplitude; i.e., the X-intercept. The intersection point on this line, determined by the solid circles, represents a control $E_{EPP}$ of -11.1 mV; treatment with theophylline moved the $E_{EPP}$ to a more positive value -5.0 mV (open circles) approximately a 6 mV difference. In Figure 26, the solid lines indicate a control $E_{EPP}$ value of -5.7 mV obtained in another cell (solid circles, solid line). After ten minutes treatment with 1.8 mM theophylline, the equilibrium potential shifted to +0.74 mV (open circle, solid line). The dashed lines represent an experiment performed in the same cell after thirty minutes rinsing with normal Ringer solution. The second control $E_{EPP}$ was -9.6 mV (solid circles, dashed line). Theophylline treatment shifted the $E_{EPP}$ to +2.5 mV (open circles, dashed line). Thus, as shown in these experiments, theophylline shifted the equilibrium potential to a more positive
Figure 25. Effect of 1.8 mM theophylline on equilibrium potential of the EPP. Control = solid circles ($E_{EPP}=-11.1$ mV); treatment with 1.8 mM theophylline = open circles ($E_{EPP}=-5.0$ mV).
Figure 26. Effect of 1.8 mM theophylline on the equilibrium potential of the EPP. Control = solid line, solid circles ($E_{EPP} = -5.7$ mV); treatment with 1.8 mM theophylline = solid line, open circles ($E_{EPP} = +0.73$ mV). After rinsing with normal Ringer solution for 30 minutes, post-treatment control = dashed line, solid circles ($E_{EPP} = -9.6$ mV); retreatment with 1.8 mM theophylline = dashed line, open circles ($E_{EPP} = +2.5$ mV).
level and the change was both reversible and repeatable.

The alteration in the $E_{EPP}$ did produce an EPP of larger amplitude by direct measurement. This effect is illustrated in Figure 27. At the same membrane potential of $-38 \text{ mV}$, the control EPP (left tracing) increased in amplitude after treatment with theophylline (right tracing).

In this experiment, the control $E_{EPP}$ was determined to be $+0.46 \text{ mV}$ by linear regression extrapolation. Treatment with theophylline moved the $E_{EPP}$ to a more positive value of $+6.15 \text{ mV}$. This value is in excellent agreement with the value of $+6.0 \text{ mV}$ obtained by direct measurement. At this membrane potential, i.e. the equilibrium potential, the inward sodium current is balanced by the outward potassium current. As a result, an EPP is not generated in response to nerve stimulation. This phenomenon is illustrated in the second trace on the right of Figure 27. Further depolarization of the membrane potential results in an inversion of the EPP due to a proportionately greater potassium current (top right trace).

A current-voltage plot from a typical experiment in cut muscle fibers is graphed in Figure 28. As in the uncut muscle, treatment with theophylline resulted in a decreased membrane resistance, determined by the decreased slope of the regression line (open circles). Table 12 summarizes the effect of theophylline on membrane characteristics in the cut muscle fiber preparation from eleven experiments (8 cats). The $E_{EPP}$ is significantly shifted to a more positive level after treatment.
Figure 27. Equilibrium potential determination by direct measurement. Tracing 2 and 3 on the left and 1, 2, 3, and 5 on the right are electrotonic potentials induced by increasing current pulses. In the middle of these electrotonic potentials an EPP has been evoked. The numbers on the left represent the membrane potentials of those EPPs. The first tracing on the left is the control EPP; the fourth tracing on the right is an EPP after 10 minutes treatment with 1.8 mM theophylline. Calibration = 5 mV x 1 msec.
Figure 28. Effect of 1.8 mM theophylline on membrane resistance in cut muscle fiber. Control = solid circles; treatment with 1.8 mM theophylline = open circles.
TABLE 12

EFFECT OF 1.8 mM THEOPHYLLINE ON MEMBRANE CHARACTERISTICS IN CUT FIBER PREPARATIONS

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± S.E.) (N = 11)</th>
<th>Treated (mean ± S.E.) (N = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium Potential ($E_{EPP}$) (mV)</td>
<td>10.87 ± 2.87</td>
<td>4.91 ± 2.20*</td>
</tr>
<tr>
<td>Input Resistance (R) ($\times 10^5$ ohms)</td>
<td>1.55 ± .21</td>
<td>1.27 ± .16*</td>
</tr>
<tr>
<td>Resistance of a unit area ($R_m$) (ohm cm$^{-2}$)</td>
<td>984 ± 266</td>
<td>692 ± 181*</td>
</tr>
<tr>
<td>Resting Potential (R.P.) (mV)</td>
<td>34.0 ± 2.5</td>
<td>22.0 ± 1.9*</td>
</tr>
<tr>
<td>Diameter (d) (µ)</td>
<td>75 ± 9</td>
<td>75 ± 9</td>
</tr>
</tbody>
</table>

*Statistically significant difference ($p < 0.05$) between paired control and treated values, one tailed t-test
Membrane resistance, $R$, resistance for a unit area of membrane, $R_m$, and resting membrane potential, $R_P$, are all significantly decreased.

Due to the change in $E_{EPP'}$, it was of interest to re-evaluate the data obtained from trains of EPP investigating presynaptic parameters (Tables 6 & 7). Adjustments were made in Martin's correction for non-linear summation to account for changes in $E_{EPP}$.

In the cut fiber preparation, an $E_{EPP}$ of $-11$ mV was used for control data and an $E_{EPP}$ of $-5$ mV for theophylline treatment. The recalculated parameters are summarized in Table 13. Quantal content, probability of release, and mobilization are significantly increased, while quantum size significantly decreased after making this adjustment.

The quantal analysis data remained unaltered when the trains of EPPS obtained in dTc-blocked preparations were subjected to a change in equilibrium potential (Tables 6, 14). In this case, the control $E_{EPP}$ used was $-15$ mV as reported by Takeuchi and Takeuchi (1959) for dTc-blocked muscle. The theoretical theophylline $E_{EPP}$ used was $-9$ mV, since the absolute difference in $E_{EPP}$ in the cut fiber preparation was $6$ mV.

Theophylline altered both the passive membrane (resistance) and activated membrane (equilibrium potential) characteristics. These membrane phenomena contribut to the observed amplitude of MEPPS, and ACh or carbachol depolarizations (Tables 2, 9 & 10). The degree
### TABLE 13

**EFFECT OF 1.8 mM THEOPHYLLINE ON NEUROMUSCULAR TRANSMISSION IN CUT FIBER PREPARATIONS WITH CORRECTED EQUILIBRIUM POTENTIALS**

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_{\text{EPP}} = -11\text{mV} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude of First EPP (mV)</td>
<td>15.0 ± 3.4</td>
<td>15.8 ± 3.7</td>
</tr>
<tr>
<td>Quantum Size (q)</td>
<td>0.062 ± 0.011</td>
<td>0.060 ± 0.020</td>
</tr>
<tr>
<td>Quantal Content (Mo)</td>
<td>243 ± 23</td>
<td>337 ± 29*</td>
</tr>
<tr>
<td>Mobilization (q/msec)</td>
<td>22.32 ± 1.84</td>
<td>26.28 ± 2.17*</td>
</tr>
<tr>
<td>Available Stores (N)</td>
<td>4359 ± 442</td>
<td>4014 ± 384</td>
</tr>
<tr>
<td>Probability of Release (p)</td>
<td>0.056 ± 0.006</td>
<td>0.087 ± 0.009*</td>
</tr>
</tbody>
</table>

\( N=7; \) stimulation frequency = 200/sec.

*Statistically significant difference (\( p < 0.05 \)) between paired control and treated values, one tailed t-test.
**TABLE 14**

**EFFECT OF 1.8 mM THEOPHYLLINE ON NEUROMUSCULAR TRANSMISSION IN d-TUBOCURARINE BLOCKED PREPARATIONS WITH CORRECTED EQUILIBRIUM POTENTIALS**

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{EPP} = -15\text{mV}$</td>
<td>$E_{EPP} = -9\text{mV}$</td>
</tr>
<tr>
<td>Amplitude of First EPP (mV)</td>
<td>2.67 ± 0.39</td>
<td>3.49 ± 0.41*</td>
</tr>
<tr>
<td>Quantum Size (q)</td>
<td>.007 ± .001</td>
<td>.010 ± .002*</td>
</tr>
<tr>
<td>Quantal Content (Mo)</td>
<td>396 ± 64</td>
<td>392 ± 69</td>
</tr>
<tr>
<td>Mobilization (q/msec)</td>
<td>7.81 ± 1.20</td>
<td>7.34 ± 1.05</td>
</tr>
<tr>
<td>Available Stores (N)</td>
<td>2045 ± 305</td>
<td>2013 ± 274</td>
</tr>
<tr>
<td>Probability of Release (p)</td>
<td>.199 ± .015</td>
<td>.194 ± .017</td>
</tr>
</tbody>
</table>

N=7; stimulation frequency = 50/sec.

*Statistically significant difference ($p < 0.05$) between paired control and treated values, one tailed t-test.
of the subsynaptic permeability change (G) in the presence of these altered membrane characteristics may be much greater than the voltage change observed. Therefore, it is necessary to compare the conductance changes produced by these potentials (see Calculations). Table 15 analyzes the effect of theophylline on the conductance changes produced by MEPPS, ACh, and carbachol depolarizations. In all cases, the conductance increased after theophylline treatment.


**TABLE 15**

**EFFECT OF 1.8 mM THEOPHYLLINE ON THE CONDUCTANCE CHANGE PRODUCED BY MEPPS AND ACh AND CARBACHOL DEPOLARIZATIONS**

<table>
<thead>
<tr>
<th></th>
<th>MEPP (10^-8 mho)</th>
<th>ACh Depolarization (10^-8 mho)</th>
<th>Carbachol Depolarization (10^-8 mho)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frog</td>
<td>Cat</td>
<td>Frog</td>
</tr>
<tr>
<td>Control</td>
<td>2.8</td>
<td>5.9</td>
<td>12.6</td>
</tr>
<tr>
<td>Treated</td>
<td>4.9</td>
<td>10.9</td>
<td>29.4</td>
</tr>
<tr>
<td>Percent Increase (%)</td>
<td>75.0</td>
<td>84.74</td>
<td>133.33</td>
</tr>
</tbody>
</table>

Statistical analysis not performed since data was calculated from mean values
CHAPTER IV

DISCUSSION
CHAPTER IV

DISCUSSION

Theophylline possesses both pre- and postsynaptic facilitatory actions at the cat neuromuscular junction. These actions are novel and have not previously been described in detail for other mammalian species.

Effect of Theophylline on MEPPS

Results obtained from experiments on spontaneously recorded MEPPS demonstrate that theophylline exerts the following effects:
1) MEPP frequency is increased approximately 200%; 2) the amplitude of the MEPPS is increased about 30%; and 3) the time course of the MEPP is prolonged (rise-time about 25% and 1/2 fall about 30%).

Caffeine has been reported to increase MEPP frequency in rat diaphragm (Elmqvist and Feldman, 1965; Hofmann, 1969) and in frog sartorius muscle (Mambrini and Benoit, 1963). Goldberg and Singer (1969) observed that theophylline also increased MEPP frequency. These investigators suggested that the increased frequency was due to: 1) unbinding of Ca presynaptically (Elmqvist and Feldman, 1965); 2) increasing calcium efflux postsynaptically (Bianchi, 1961), thereby increasing extracellular Ca concentration (Mambrini and Benoit, 1963);
and 3) increasing cyclic AMP in the nerve terminal resulting in altered calcium levels (Singer and Goldberg, 1970). There is insufficient data reported to attribute a specific mechanism to this facilitatory action of theophylline, although the evidence does suggest that it may be calcium linked.

In contrast to the results obtained in the present study, Goldberg and Singer (1969) reported that 1.8 mM theophylline treatment did not produce any change in MEPP amplitude. Mambrini and Benoit (1963) found, however, that caffeine produced a slight increase in MEPP amplitude which was not statistically significant. An investigation by Ginsborg and Hirst (1972) confirmed the results of the present study (Shinnick and Jacobs, 1971; 1972) since they reported that 1.8 mM theophylline produced about a 30% increase in MEPP amplitude.

The increased amplitude of MEPPs may be due to either a pre- or postsynaptic effect, through the following possible mechanisms: 1) a MEPP composed of multiple quantal units resulting from the marked increase in frequency; 2) a presynaptic increase in the unit size of transmitter; 3) a postsynaptic increase in membrane resistance; 4) cholinesterase inhibition; and 5) a change in the equilibrium potential of the transmitter at the subsynaptic membrane. As seen on the histogram (Figure 10), there is a shift of the MEPP amplitude distribution to the right after theophylline treatment with a corresponding increase in the incidence of larger MEPP amplitudes. An increase
in MEPP amplitude due to summation should show another large peak occurring at 1.0 mV while maintaining essentially the same amplitude distribution pattern. Figure 11 illustrates that MEPPS do not appear to summate after theophylline treatment, since partially summated MEPPS (i.e. a second MEPP superimposed on the rising or falling phase of a first MEPP) were not observed or recorded. It can not be concluded from these experiments whether the effect of theophylline is totally presynaptic or has a postsynaptic component.

The ethylenediamine salt does not appear to influence the effect of theophylline. Ethylenediamine does seem to contribute to respiratory stimulation (Marais and McMichael, 1937) and to the inotropic action of aminophylline in isolated hearts (Hardman, Yard, and Chenoweth, 1953). Clinical evidence suggests that this inotropic effect only occurs to a slight degree in man (Howarth, McMichael, and Sharpey-Schafer, 1947). Similarly, Huidobro (1945) did not attribute any experimental results obtained on muscle contraction in cat quadriceps femoris muscle to ethylenediamine since there was little difference between the theophylline base and the theophylline ethylenediamine salt. Thus, in skeletal muscle, the effect of ethylenediamine in relation to that of theophylline appears to be minimal.
Effect of Theophylline on MEPP and EPP Time Course

In addition to increasing the amplitude, theophylline prolongs the rise-time and $1/2$ fall of MEPPs and of EPPs in dTc-blocked muscle and in cut muscle fiber preparations. An increase in time course may be attributed to one of several factors; i.e. 1) inhibition of cholinesterase, thereby allowing ACh to react with the receptor for a longer period of time; 2) an increase in the time constant of the membrane or 3) a slowed release of transmitter due to hyperpolarization of the nerve terminals.

A hyperpolarizing current applied to the nerve terminals increases the amplitude and prolongs the rise-time but has no effect on the $1/2$ fall of EPPs (Hubbard and Willis, 1962). A hyperpolarization of the nerve terminal would also decrease MEPP frequency (Liley, 1956c) contrary to the increase seen with theophylline treatment. Thus, the prolongation of MEPP and EPP time course may not be due to a presynaptically induced hyperpolarization of the nerve terminal.

Mambrini and Benoit (1963) reported that the time course of EPP in a dTc-blocked preparation with caffeine treatment is prolonged too slightly to be due to cholinesterase inhibition. However, their conclusion is not supported by any experimental evidence.
Effect of Theophylline on Trains of EPPS

In the dTc-blocked fiber, the presynaptic release parameters are not altered; i.e. probability of release, quantal content, mobilization, available stores are unaffected by theophylline treatment. Mambrini and Benoit (1963) reported that quantal content in high Mg Ringer solution, though never decreased, was never uniformly increased by caffeine. Using the quantal analysis in dTc-blocked muscle, Hofmann (1969) found that caffeine reduced probability of release without any change in quantal content. This investigator did not find any change in MEPP amplitude in unpaired experiments; however, in a few paired experiments he reported an increase in EPP variability (therefore increasing quantum size). These data were discarded as artifact. Due to the wide variability between fibers, paired experiments are mandatory to validly apply the quantal analysis (Elmqvist and Quastel, 1965b). Hofmann (1969) concluded that caffeine increased the rate of transmitter replenishment. Singer and Goldberg (1970) reported that theophylline increased quantal content in dTc-immobilized rat diaphragm preparations. They attributed their differing results to the frequency of stimulation used in Hofmann's (1969) experiments.

Hubbard, Wilson, and Miyamoto (1969) using the rat diaphragm reported that dTc greatly increased the probability of release. Similar results have been found in our laboratory (Blaber, 1970;
Jacobs and Blaber, 1971; Gallagher and Blaber, 1973) and in this present study with theophylline (c.f. Control, Tables 6 & 7) in cat tenuissimus muscle. Thus, alternatively, dTc may increase probability of release in a manner that theophylline may not be able to antagonize.

Results obtained in cut muscle fiber preparations have demonstrated that the effect of theophylline on release parameters is unmasked in the absence of dTc, since quantal content and probability of release are increased while available stores is decreased.

An increased probability of release may be due to alterations in nerve action potentials (Hagiwara and Tasaki, 1958; Katz and Miledi, 1965c), mobilization of calcium in the nerve terminal and the concomitant increased probability of release (Katz and Miledi, 1965b; Hubbard, Jones, and Landau, 1968b) or a direct effect on excitation-secretion coupling mediated by cyclic AMP (Breckenridge, Burn, and Matschinsky, 1967; Goldberg and Singer, 1969).

Goutier (1949b) reported that theophylline treatment did not modify the action potential of the frog sciatic nerve preparation. This evidence suggests that a change in the nerve action potential may not be responsible for the increase in probability of release seen with theophylline.

In isolated human muscle raising calcium concentration produces the same results, including an increase in mobilization (Elmqvist and Quastel, 1965b). Using the cut muscle fiber preparation, Wilson
and DeVillez (1973) recently reported that a double concentration of calcium produces an increase in the probability of release and mobilization but decreases the available store, results identical to this present study. The dibutyl cyclic AMP derivative, similarly increases probability of release and mobilization; however, this compound increases the readily available stores. These authors concluded that cyclic AMP had two sites of action in the nerve terminal: 1) it affects the intracellular store of calcium resulting in an increase in probability of release and mobilization, and 2) it has a metabolic effect resulting in an increase in the releasable stores. The effects of theophylline seem to be independent of phosphodiesterase inhibition, since if its action were mediated through cyclic AMP, an increase of the available stores should be observed. Theophylline appears to have an action identical to that produced by calcium.

**Effect of Theophylline on EPP Amplitude**

In dTc-blocked fibers and the cut muscle fiber preparation, EPP amplitudes are increased with theophylline treatment. Goldberg and Singer (1969) attributed the increased EPP amplitude with theophylline to a presynaptic phenomenon, an increase in the number of quanta in an EPP (quantal content). Mambrini and Benoit (1963), however, did not find a consistent increase in quantal content with caffeine using high Mg Ringer solution. They concluded that the increase in EPP amplitude
was due to an increased sensitivity of the ACh receptor. The increased quantal content observed in cut fiber experiments may be due to the increased probability of release, since quantum size was not found to increase. Furthermore, I observed a reduction in the amplitude of the 95th EPP in the tail and this may be associated with the marked increase in probability of release without sufficient increase in mobilization to compensate. In any case, there is insufficient evidence to attribute the increased EPP amplitude solely to a presynaptic increase in quantal content. The increased MEPP amplitude suggests that theophylline may possess a postsynaptic site of action. The experiments on dTc-depressed MEPPS further supports this view.

Effects of Theophylline on dTc-Depressed MEPPS

MEPP amplitude, significantly depressed by d-tubocurarine, returned to control values after treatment with theophylline. One explanation for the reversal of the dTc-depressed MEPPS is an antagonism of the postsynaptic action of dTc. However, this effect may also be achieved with a presynaptic increase in the unit size of transmitter, i.e. the quantum. These two hypotheses were examined in experiments using exogenously applied ACh.

Iontophoretic Application of ACh

The results obtained from the experiments on iontophoretically
applied ACh were inconsistent. The following observations were made: 1) many times the cell was lost when theophylline was added to the perfusion media; and 2) MEPPs increased in amplitude during theophylline treatment while the ACh potential remained unchanged. These results could indicate that theophylline may increase MEPP amplitude through a presynaptic mechanism. On the other hand, Mambrini and Benoit (1963) observed that caffeine potentiated the response to iontophoretically applied ACh in a reversible manner. Since this change was not accompanied by important changes in duration, they concluded that caffeine increased the sensitivity of the endplate. In contrast, Magazanik and Vyskocil (1970) reported that caffeine did not influence the amplitude of the ACh potentials.

There are reports of movement artifact with iontophoretically applied ACh (Takeuchi, 1963; Thesleff, 1959) that may explain the differences in the reported data. Thesleff applied ACh pulses to a preparation prone to spontaneous movement, i.e. hypertonic NaCl. As a result, his experimental interpretation proved wrong (Otsuka, Endo, and Nonomura, 1962). Similarly, in high calcium Ringer solution, Takeuchi (1963) reported visible shortening of the muscle fiber when subthreshold ACh potentials were applied. It is well established that methylxanthines increase the influx of Ca ions (Bianchi, 1961). It is possible that the methylxanthines under the action of ACh increase the influx of Ca ions. Subsequently, the
contractile mechanism may be activated to produce localized muscle shortening, thereby altering the distance between the recording and ACh electrode. This phenomenon would yield unpredictable results. Therefore, I decided to use a technique which would minimize the possibility of interference due to movement artifact.

Effect of Theophylline on ACh Depolarizations

ACh responses in both the frog and cat muscle were facilitated by theophylline. Experiments on muscle contraction indicate that theophylline may act postsynaptically; however, these experiments cannot differentiate an action on the subsynaptic membrane from a direct action on muscle contraction. For example, Torda and Wolff (1945) found that theophylline enhanced an ACh contracture on the frog rectus abdominis muscle. Huidobro (1945) likewise observed that caffeine augmented the response to ACh in denervated muscle. From the results of the present study it can be concluded that theophylline enhances an ACh response through an action on the subsynaptic membrane. The postsynaptic increases in the ACh response coupled with the prolongation of MEPP and EPP time course is characteristic of cholinesterase inhibition. For this reason, the choline ester carbachol was used since it is not influenced by cholinesterase.
Effect of Theophylline on Carbachol Responses

In both the frog and cat theophylline treatment resulted in a slight facilitation of the carbachol depolarizations which was not statistically significant. Thus, these experiments indicate that theophylline may increase MEPP and EPP amplitude and time course by inhibiting acetylcholinesterase. Although the substitution of carbachol for ACh is the best experimental method for an electrophysiological estimate of possible anticholinesterase activity, it is not entirely conclusive for the following reasons: 1) carbachol is not the transmitter at the cat neuromuscular junction; 2) Katz and Miledi (1972) have recently calculated that an ion channel opened by carbachol molecules stays open for only 0.3-0.4 msec compared with 1 msec when activated by ACh; 3) there is a slight potentiation of the carbachol depolarization with theophylline noted in all experiments; and 4) the depolarizations produced by carbachol are more variable than ACh depolarizations. Since these experiments did not provide conclusive evidence that theophylline increased MEPP and EPP amplitude and time course by solely inhibiting acetylcholinesterase, experiments were performed to investigate other mechanisms which may be responsible for these effects.

Passive Membrane Characteristics

The membrane resistance (R), and the time constant (T_m) decreased
with theophylline treatment accompanied by a decrease in the resting membrane potential. In all experiments in this study, theophylline treatment resulted in a slight, consistent depolarization of the resting membrane potential, opposite to the observations of Ginsborg and Hirst (1972). It has been observed that caffeine treatment produced a similar decrease in membrane resistance with a concomittant decrease in resting membrane potential (Axelsson and Thesleff, 1958).

Theophylline has been shown to prevent Ca binding in isolated sarcoplasmic reticulum (Johnson and Inesi, 1969). Since muscle membrane resistance is increased in high Ca Ringer solution (Tamashige, 1951; Jenerick, 1959), the prevention of Ca binding may be the cause of the decreased membrane resistance. Calcium lack has also been shown to produce a resting membrane potential depolarization (McIntyre, Young and Ware, 1956). Thus, Ca unbinding may be responsible for the membrane depolarization which occurred with theophylline treatment.

From these experiments it can be concluded that: 1) since the decreased time constant would not account for the prolongation of MEPP and EPP time course produced with theophylline, cholinesterase inhibition may be responsible; 2) a decreased resistance would result in an MEPP and EPP of smaller amplitude, therefore, could not account for the increase in amplitude seen with theophylline treatment. In fact, the absolute increase in MEPP and EPP amplitude would be masked by the decreased resistance. Because these experiments did not resolve the question of the increased amplitude of the MEPP and EPP, the equilibrium potential of the EPP was examined.
Equilibrium Potential of the Endplate Potential

Theophylline shifted the equilibrium potential to a more positive level and thus the driving force \((RP - E_{EPP})\) for the EPP increased causing an increase in EPP amplitude.

Theophylline has been shown to: 1) possess presynaptic actions, 2) inhibit cholinesterase, 3) antagonize a dTc block. The question should then be raised as to whether these actions may interfere with the \(E_{EPP}\) measurements. Catechol, a drug which acts to augment the release of acetylcholine and reverse a dTc block by a totally presynaptic action, does not alter the equilibrium potential (Gallagher and Blaber, 1973). Edrophonium, a short-acting anti-ChE agent, does not change the \(E_{EPP}\) (Blaber, 1972). Therefore, cholinesterase inhibition probably is also not involved. Simple competition with dTc should not theoretically produce a shift as dTc itself has no known effect on the \(E_{EPP}\) (Takeuchi and Takeuchi, 1968). Thus, the evidence suggests that the shift in \(E_{EPP}\) produced by theophylline is independent of its other actions.

An alteration of the \(E_{EPP}\) has been shown to occur with only one other substance – calcium (Takuechi, 1963). Takeuchi (1963) found that increased extracellular concentrations of calcium shifted the \(E_{EPP}\) to a more negative value by selectively decreasing the sodium conductance. Theophylline may be producing a response similar to lowering calcium concentrations, since the shift in the \(E_{EPP}\) is in
the opposite direction, i.e. to a more positive value. This shift may be due to a theophylline-induced dissociation of calcium in the vicinity of the endplate that can mimic the lowering of extracellular Ca concentration. It has also been shown in squid axon that the increase in excitability in low calcium is caused by an increase in sodium conductance (Weidmann, 1955; Frankenhauser and Hodgkin, 1957; Frankenhauser, 1957). In fact, Hodgkin (1958) suggested that the calcium ions absorbed on the membrane influence its permeability and excitability. Thus, by unbinding calcium, theophylline may allow more sodium to enter the sub­synaptic membrane increasing $g_{Na}$ and shifting the $E_{EPP}$ to a more positive level.

A similar situation may exist in the electrically excitable muscle membrane, where calcium regulates Na permeability (Koketsu, 1969). By un­binding Ca theophylline may allow more sodium to enter the cell producing the resting membrane depolarization observed in all experiments.

Since the equilibrium potential was used in the quantal analysis experiments, these data were re-evaluated. When the control values in the cut muscle fiber preparation are subjected to a change in equilibrium potential (i.e. from -15 mV to -11 mV; c.f. control, Table 16), all the presynaptic parameters are altered. These results indicate that a constant value for $E_{EPP}$ in correcting for non-linear summation should not be used, since it is probable that the intracellular ionic concentrations of each cell in the cut muscle fiber preparation vary
### TABLE 16

**SUMMARY OF THE EFFECT OF 1.8 mM THEOPHYLLINE ON NEUROMUSCULAR TRANSMISSION IN CUT FIBER PREPARATIONS**

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
<th>Control (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{EPP}$ = -15mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude of First EPP (mV)</td>
<td>16.78 ± 4.45</td>
<td>24.19 ± 7.45</td>
<td>15.0 ± 3.4</td>
<td>15.8 ± 3.7</td>
</tr>
<tr>
<td>Quantum Size (q)</td>
<td>.070 ± .014</td>
<td>.068 ± .013</td>
<td>.062 ± .011</td>
<td>.060 ± .020</td>
</tr>
<tr>
<td>Quantal Content ($M_o$)</td>
<td>238 ± 22</td>
<td>340 ± 35*</td>
<td>243 ± 23</td>
<td>337 ± 29*</td>
</tr>
<tr>
<td>Mobilization (q/msec)</td>
<td>20.92 ± 1.73</td>
<td>21.37 ± 1.46</td>
<td>22.32 ± 1.84</td>
<td>26.28 ± 2.17*</td>
</tr>
<tr>
<td>Available Stores (N)</td>
<td>4025 ± 439</td>
<td>3237 ± 307*</td>
<td>4359 ± 442</td>
<td>4014 ± 384</td>
</tr>
<tr>
<td>Probability of Release (p)</td>
<td>.062 ± .007</td>
<td>.112 ± .017*</td>
<td>.056 ± .006</td>
<td>.087 ± .009*</td>
</tr>
</tbody>
</table>

N=7; stimulation frequency = 200/sec.

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test*
greatly from fiber to fiber. The variation in $E_{EPP}$ from fiber to fiber coupled with the theophylline induced change in $E_{EPP}$ may have masked a postsynaptic increase in quantum size ($q$) determined by quantal analysis that is readily seen by direct measurement of MEPPS. Since all these parameters are dependent on the calculation of $q$ (see Calculations), any alteration of $q$ changes all dependent variables. Table 16 which compares the effect of shifting $E_{EPP}$ by 4 mV (c.f. Controls) shows that probability of release is decreased and available stores is increased by this substitution. Since, in fact, theophylline treatment increases probability of release and decreases available stores before and after correction of the $E_{EPP}$, these presynaptic changes probably represent a true drug effect.

In contrast, in dTc-blocked fibers a change in $E_{EPP}$ produced no alterations in the interpretation of the quantal analysis (Table 17). It seems that a cell in a dTc-blocked fiber is less variable in its intracellular ionic state than the cut muscle fibers, since in the dTc-blocked preparation, increase in estimates of $q$ parallel the increase in MEPP amplitude seen with theophylline treatment.

In MEPPS, ACh and carbachol depolarizations, conductance increases after theophylline treatment. Since the change in conductance produced by theophylline with ACh potentials is greater than that with carbachol, some of the increase in MEPP amplitudes may be attributed to cholinesterase inhibition which may account for the prolonged time
TABLE 17
SUMMARY OF THE EFFECT OF 1.8 mM THEOPHYLLINE ON NEUROMUSCULAR TRANSMISSION IN d-TUBOCURARINE BLOCKED PREPARATIONS

<table>
<thead>
<tr>
<th></th>
<th>Control (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
<th>Treated (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude of First EPP (mV)</td>
<td>2.67 ± 0.39</td>
<td>3.51 ± 0.41*</td>
<td>3.49 ± 0.41*</td>
</tr>
<tr>
<td>Quantum Size (q)</td>
<td>.007 ± .001</td>
<td>.010 ± .002*</td>
<td>.010 ± .002*</td>
</tr>
<tr>
<td>Quantal Content (Mo)</td>
<td>396 ± 64</td>
<td>389 ± 69</td>
<td>392 ± 69</td>
</tr>
<tr>
<td>Mobilization (q/msec)</td>
<td>7.81 ± 1.20</td>
<td>7.26 ± 1.05</td>
<td>7.34 ± 1.05</td>
</tr>
<tr>
<td>Available Stores (N)</td>
<td>2045 ± 305</td>
<td>1991 ± 273</td>
<td>2013 ± 274</td>
</tr>
<tr>
<td>Probability of Release (p)</td>
<td>.199 ± .015</td>
<td>.195 ± .017</td>
<td>.194 ± .017</td>
</tr>
</tbody>
</table>

N=7; stimulation frequency = 50/sec

*Statistically significant difference (p < 0.05) between paired control and treated values, one tailed t-test
course of MEPPS and EPPS. The residual conductance change, independent of cholinesterase inhibition, may be caused by a shift in the equilibrium potential.

In conclusion, aside from the presumed effect on cholinesterase, the actions of theophylline seem to possess one underlying factor linking pre- and postsynaptic sites of action. To quote Bernard Katz (1966):

It is interesting to note certain features that the initiation of muscle contraction shares with the initiation of an action potential and with the release of transmitter substance at a nerve terminal. In all three cases, the primary event is a depolarization of the cell membrane. Depolarization, however, is not a sufficient stimulus; it becomes effective in producing its diverse results only if calcium is present, whether the final result be increase of sodium permeability (Hodgkin, 1958), the facilitated release of acetylcholine quanta (Katz, 1962) or the activation of myosin (Huxley, 1964).

Thus, theophylline may through a common mechanism increase MEPP frequency and probability of release, decrease membrane resistance, and shift the equilibrium potential - its ability to mobilize calcium.

CONCLUSIONS

The sites and possible mechanisms of action of theophylline were investigated at the neuromuscular junction of cat tenuissimus muscle using intracellular microelectrode techniques. Analysis of the data resulted in the following conclusions:

1) theophylline increased MEPP frequency, indicating a pre-synaptic site of action;
2) in cut muscle fiber preparations, theophylline facilitated evoked release presynaptically by increasing probability of release and decreasing readily releasable stores;

3) in dTc-immobilized preparations, presynaptic facilitation was not observed;

4) MEPP and EPP amplitude increased in all preparations;

5) MEPP and EPP time course was prolonged;

6) a postsynaptic site of action was identified, since theophylline potentiated responses to exogenously applied ACh;

7) since the carbachol response was potentiated to a lesser extent than the ACh response, cholinesterase inhibition was suggested;

8) cholinesterase inhibition may also be responsible for the prolonged rise-time and 1/2 fall of the MEPP and EPP;

9) theophylline produced a depolarization of the resting membrane potential in all experiments;

10) theophylline decreased membrane resistance in normal and in transversally cut muscle fiber preparations;

11) the decreased membrane resistance with the concurrent increase in the ionic permeability of the membrane may account for the resting potential depolarization;

12) theophylline shifted the equilibrium potential of the EPP to a more positive value in transversally cut muscle fibers;

13) the altered $E_{EPP}$ coupled with cholinesterase inhibition
may account for the increase in EPP and MEPP amplitude and prolonged time course;

14) these effects were explained through a possible calcium mechanism.
BIBLIOGRAPHY


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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dec. 19, 1973
Date

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