1971

Blockade of Acetylcholine Receptors by Cobra Toxin Electrophysiology and Pharmacology

Henry A. Lester

BLOCKADE OF ACETYLCHOLINE RECEPTORS BY COBRA TOXIN: ELECTROPHYSIOLOGY AND PHARMACOLOGY

A thesis submitted to the Faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

Henry A. Lester, A.B.

Approval for publication

F. A. Dodge Jr

April 1, 1971
The Rockefeller University
New York

ACKNOWLEDGMENTS

A graduate education in twentieth-century science is quite a cosmopolitan experience. One need only glance through the list of references to convince oneself that this field, in particular, receives contributions from a large international community.

It is therefore fitting that my own education has profited from mentors in several different locations. At the Rock, Fred Dodge has provided daily advice and guidance. Floyd Ratliff is also a kind, insightful advisor. I also thank H. K. Hartline for generous support, Alex Mauro for friendship and scholarship, Bruce Knight for help with mathematical analyses, and C. M. Connelly and Dave Mauzerall for introducing me to the study of diffusion processes. Mura Lipski, Norman Milkman, Louis Nagy, Marie Azzarello, and Teresa Colaco have provided technical and clerical help; Mary Costello patiently and expertly typed this thesis.

At Woods Hole, Mike Bennett and Larry Cohen taught me how to work with electroplaque preparations, and Felix Strumwasser introduced me to Aplysia. John Hervey taught me that there is at least one right way to do everything.

At Cambridge, Victor Whittaker and Roger Marchbanks tutored me in the chemistry of the nervous system.

Dave Eaker of the Biochemical Institute at Uppsala has faithfully provided large quantities both of his purest cobra toxins and of excellent background information.

Finally, warm thanks to my skiing, sailing, and squash partners over the past five years.

ABSTRACT

Cholinergic transmission has been studied in the presence of a pure polypeptide toxin, of known amino-acid sequence, from cobra venom. For the frog sartorius myoneural junction, micropipette electrodes and on-line computer were used to show that the toxin specifically and irreversibly inactivates postsynaptic acetylcholine (ACh) receptors. Raja and Torpedo electroplaques also show nicotinic pharmacology and were similarly affected by the toxin. The toxin failed to affect the muscarinic receptors of the isolated frog heart or the receptors of Aplysia neurons (neither nicotinic nor muscarinic). These and other observations suggest that the toxin's pharmacological specificity is confined to nicotinic ACh receptors.

Further studies were conducted with the frog myoneural junction. At concentrations greater than 10^{-8} M, the toxin irreversibly inactivated receptors with first-order kinetics. The rate constant for inactivation increased linearly with the toxin concentration near the receptors.

Micro-ionophoretic application of two reversible agonists, ACh and carbamylcholine (Carb), focally protected receptors against bath-applied toxin. Bath-applied Carb and d-tubocurarine (dTC) (a reversible antagonist) also protected against bath-applied toxin. Since the reversible agonists and antagonists are known to bind to the ACh receptor, these observations suggest that cobra toxin also binds directly to the receptor. Choline protects only at high concentrations, in agreement with its known weak effects on ACh receptors.

Quantitative studies of protection were evaluated according to a simple scheme of transitions among available, reversibly blocked, and irreversibly blocked receptors. All agonists cause desensitization, and each desensitized receptor was completely protected against the toxin. Agonists also caused departures from first-order

kinetics of receptor inactivation; these are explained by the transition scheme and known rate constants for desensitization. Protection by dTC, however, was less than would be expected if curarized receptors were completely inaccesible to the toxin. Desensitized and curarized receptors therefore have different vulnerability to the toxin; this suggests that agonists and antagonists produce fundamentally different effects on ACh receptor structure.

Abbreviations

$\alpha = 1/\tau$ - rate constant for receptor inactivation (see p. 51).

ACh - acetylcholine

Carb - carbamylcholine

D_m - diffusion constant for a substance within the muscle

dTC - d-tubocurarine

EPP - endplate potential

m - quantal content (see Martin, 1966)

$m_o = \log \frac{\text{number of stimuli}}{\text{number of failures}}$

$m_1 = \frac{\text{mean EPP amplitude}}{\text{mean MEPP amplitude}}$

$m_2 = \frac{1}{(cv)^2}$, where cv is the coefficient of variation of EPP amplitude distribution

MEPP - miniature endplate potential

MPSP - miniature postsynaptic potential

PSP - postsynaptic potential

τ - time constant for receptor inactivation (see p. 51).

$[x]$ - concentration of x.

Other Mathematical Symbols

These are defined as they appear in the text, especially Chapters IV and V.

TABLE OF CONTENTS

Page

List of Tables

CHAPTER I

HISTORICAL BACKGROUND

We Need New Tools to Study Chemical Synapses

The synapse's function is to transmit nerve signals from a sensory receptor cell to a neurone, from one neurone to another, or from a neurone to a muscle fiber or other effector cell. At a chemical synapse, the presynaptic impulse causes the presynaptic terminal to release a small amount of chemical neurotransmitter. This transmitter induces changes in the postsynaptic cell's transmembrane potential, thereby influencing its impulse activity.

The chemical synapse is one of the important tools which the nervous system uses to integrate information. Every advance in our understanding of synaptic transmission may contribute to the ultimate goal of comprehending nervous function and development.

At cholinergic synapses, a starring role is played by the membrane-bound acetylcholine "receptor" (first termed a "receptive substance" by Langley, 1906). The receptor transforms the presence of acetylcholine (ACh) molecules into changes in the ionic permeability of a postsynaptic membrane. The diversity of acetylcholine's action presumably arises because many functionally different ACh receptors occur in nature, and we begin by cataloguing these differences in the next two sections. In the following section (Structure-Function Relations at the Acetylcholine Receptor), we discuss possible features of receptor structure which allow the same molecule--acetylcholine--to produce different postsynaptic effects. Next (Elapid and Hydropheid Snake Venom Neurotoxins...), we consider the bases for hoping that cobra toxin (and homologous polypeptides) will prove useful in isolating the ACh receptor--a long-sought goal. Finally (Choice of Preparations), we outline how the concepts of this chapter have helped generate experiments to determine the toxin's pharmacological specificity.

Drugs and toxins have often advanced our knowledge of synaptic transmission, as in Claude Bernard's classical studies of curare. With purely electrophysiological techniques, I have studied the curarelike postsynaptic action of a protein toxin from cobra venom. This toxin now takes its place as a chemical probe for research on cholinergic transmission.

The Acetylcholine Receptor: Drug Recognition and Ionic Permeation

In this section, we describe how ACh produces its diverse effects on nerve and muscle membranes.

The Recognition Function: Nicotinic and Muscarinic Receptors

The receptor's first function is to recognize ACh molecules. Vertebrate ACh receptors apparently perform this task in at least two different ways, because cholinergic agonists (see p. 6) fall into two overlapping groups, depending on the receptors they excite. The same classification of receptors serves to describe susceptibility to antagonists (see p. 6). Receptors are categorized as "nicotinic" or "muscarinic," depending on the agonists and antagonists to which they show sensitivity. The terminology was introduced by Dale (1914). Studying the pharmacology of the peripheral nervous system, he first described the distinction between muscarine's action, "broadly reproducing the effects of the cranial and sacral involuntary nerves, and readily paralyzed by atropine," and a "nicotine" action, "reproducing the effects of stimulating voluntary and preganglionic sympathetic nerves, and paralyzed by curare."

It required decades to complete the evidence that ACh is in fact the normal transmitter at all these muscarinic and nicotinic synapses (reviewed by Dale, 1937). Known nicotinic and muscarinic agents have since grown quite numerous, and many of the compounds have clinical use (see, for instance, Goodman and Gilman, 1965).

The Permeation Function: Depolarizing and Hyperpolarizing Ionic Currents Experimenters using intracellular microelectrode techniques have shown that ACh produces most of its effects by transiently increasing the postsynaptic membrane's permeability to ions (reviewed by Ginsborg, 1967). This second action of the receptor is thought to take place very close to the site of ACh binding. Ionic movements can influence the postsynaptic cell's activity either by excitation, due to depolarizing currents, or by inhibition due to hyperpolarizing currents. The direction of postsynaptic currents depends on the specific ions involved. Excitatory ACh receptors at the vertebrate skeletal myoneural junction produce increases in Na^+ and K^+ permeability (Takeuchi and Takeuchi, 1960). The postsynaptic current therefore drives the transmembrane potential toward a depolarized value (about -15 mv) between the Nernst equilibrium potentials for Na^+ and K^+.

Inhibitory ACh receptors, on the other hand, produce K^+ currents (Trautwein et al., 1956). Inhibitory Cl^- currents have also been detected at noncholinergic synapses (reviewed by Eccles, 1964). Equilibrium potentials for K^+ and Cl^- are more negative than the resting potential (except possibly during the action of an electrogenic sodium pump); hence, inhibitory postsynaptic potentials (IPSPs) hyperpolarize the membrane.

It is worthwhile emphasizing that the distinction between inhibitory and excitatory receptors fails to correspond to the nicotinic-muscarinic dichotomy. Both inhibitory and excitatory muscarinic receptors occur. For instance, application of ACh mimics vagal stimulation of the heart by decreasing the strength and frequency of contractions (Hunt and Taveau, 1906; Dale, 1914); but ACh also enhances activity of the walls of the alimentary canal (Dale, 1914). Only excitatory nicotinic receptors have been described thus far.

Invertebrate ACh receptors: Recognition and Permeation The general concepts of a "recognition" and a "permeation" function, which we have used to describe vertebrate ACh receptors, also apply to invertebrates. In this section, we consider these mechanisms and their variations.

A good deal of pharmacological and biophysical information has been gathered for the ACh receptors on molluscan ganglionic neurones. Furthermore, recent studies on the cellular localization of choline acetylase and acetylcholinesterase in the Aplysia abdominal ganglion (Giller and Schwartz, 1971a,b) support the notion that ACh is a natural neurotransmitter. With these results as a background, I chose Aplysia for my studies on cobra toxin and invertebrate ACh receptors. A brief review of molluscan ACh receptors, with special emphasis on Aplysia, will be presented here.

The recognition function shows some degree of variation among molluscan ACh receptors. As in the case of vertebrates, this diversity manifests itself in the range of cholinergic drugs which evoke or block postsynaptic currents. Tauc and Gerschenfeld (1962) and Strumwasser (1962) first showed that ACh hyperpolarizes some cells and inhibits others in the Aplysia abdominal ganglion. High concentrations of d-tubocurarine (dTC) inhibit both responses, but hexamethonium antagonizes only the depolarizing responses. Eserine sulfate also has different effects on the excitatory and inhibitory receptors (Tauc and Gerschenfeld, 1962).

In the pleural ganglion, pharmacological variation has been described in connection with a two-component cholinergic IPSP (Kehoe, 1967). Both components are produced by ACh and carbamylcholine; however, other nicotinic (nicotine, propionylcholine, tetramethylammonium) or muscarinic (methacholine and oxotremorine) agonists (see p. 6) produce only the early phase. Antagonists are similarly selective. The early phase is blocked by dTC or strychnine; the late component can be blocked by tetraethylammonium (Kehoe, 1969).

Very similar properties have been described for a two-component response in the abdominal ganglion (Pinsker and Kandel, 1969; Kehoe and Ascher, 1970).

These results suggest that the distinction between nicotinic and muscarinic receptors, which applies to vertebrates, has little use for describing the recognition function in molluscan ACh receptors. Perhaps the most common pharmacological test for vertebrates involves sensitivity to atropine (muscarinic receptors) or to dTC (nicotinic). However, where it has been tested, atropine inactivates Aplysia ACh receptors as well as dTC (Tauc and Gerschenfeld, 1962; Pinsker and Kandel, 1969). The drugs block only at concentrations some two orders of magnitude higher than required for vertebrate muscarinic (atropine) and nicotinic (dTC) receptors. Thus, despite the fact that atropine and dTC block Aplysia ACh receptors, the sensitivity and selectivity are much less than in vertebrate nervous systems. Although the recognition mechanism in molluscs shows interesting variations, we cannot classify these variations according to nicotinic and muscarinic categories.

The ionic permeation mechanisms also show a great deal of variety in molluscan cholinergic receptors; the ions involved--Na^+, K^+, and Cl^---seem to be the same as for vertebrates (for reviews, see Ginsborg, 1967; Blankenship et al., 1971). Permeability changes involving Cl^- can lead either to depolarizating or hyperpolarizing currents, depending on the resting potential and on the intracellular Cl^- concentration (see discussion of Nernst potential, p. 3 ; Frank and Tauc, 1964). Both IPSPs and excitatory postsynaptic potentials (EPSPs) do in fact normally occur with Cl^- currents, and the direction of current flow does depend upon intracellular Cl^- in Aplysia (Kehoe, 1967; Blankenship et al., 1971) and in other molluscs (Kerkut and Meech, 1966; Chiarandini and Gerschenfeld, 1967; Chiarandini et al., 1967).

Another hyperpolarizing response to ACh in Aplysia involves outward K^+ currents. This is the late component of the dual inhibition described above (the early component is a Cl^- current) (Kehoe, 1967).

In addition to Cl^--dependent EPSPs, Na^+-dependent EPSPs have been described in Aplysia (Blankenship et al., 1971; Kerkut and Meech, 1966).

Thus, Aplysia and other molluscs employ a wide range of recognition and permeation mechanisms in order to generate cholinergic PSPs.

Agonists, Antagonists and Desensitization In addition to the muscarinic-nicotinic classification, cholinergic drugs may conveniently be grouped according to whether they evoke postsynaptic currents themselves, or block the currents produced by other drugs. The first group are the agonists (also called activators, or cholinomimetics); the second group, the antagonists (or inhibitors, or cholinolytics).

It is thought that most antagonists in clinical use (d-tubocurarine, gallamine, atropine, and others) interfere with the receptor's recognition function. However, agonists themselves usually produce a phase of antagonism after acting on receptors for extended periods of time; and this action may involve interference with the permeation function.

The phase of antagonism produced by agonists is called desensitization. This process has received careful study at the vertebrate myoneural junction, and for this case every agonist produces desensitization.

Invertebrate ACh receptors also show desensitization (Tauc and Bruner, 1963; Bennett, 1971), although considerably fewer facts are known about the phenomenon. Agonists and antagonists and models for their action receive excessive attention in Chapter V.

Colinergic Actions Probably Unrelated to the Receptor

Acetylcholinesterase Acetylcholine is hydrolyzed by acetylcholinesterase, a membrane-bound enzyme whose function seems to be rapid termination of acetylcholine's transmitter action. Drugs such as prostigmine show anticholinesterase activity at concentrations which do not affect ACh receptors. Other drugs, such as carbamylcholine (Carb), are nearly as effective as ACh itself in activating both nicotinic and muscarinic receptors, but these drugs completely resist hydrolysis by acetylcholinesterase. Such observations show that different structural features determine acetylcholine's recognition by the ACh receptor and its hydrolysis by acetylcholinesterase.

Diffusion Barriers to Cholinergic Drugs Some operational distinctions among cholinergic drugs derive from mechanisms secondary to direct membrane actions. For instance, both dihydro-β-erythroidine and dTC block transmission at the myoneural junction; when applied systemically, only dihydro-β-erythroidine blocks the motoneuron-Renshaw cell synapse (Eccles et al., 1954, 1958). However, micro-ionophoretic applications of dTC do block this synapse (Curtis and Eccles, 1958). It appears that systemically applied dTC is excluded from these synapses by an extracellular permeability barrier between the blood and the extracellular fluids of the central nervous system (Davson, 1957). An additional diffusion barrier seems to exist in the immediate vicinity of the Renshaw cell. This barrier lengthens the excitatory action of ACh by delaying its diffusion away from the postsynaptic membrane (Curtis and Eccles, 1958).

Other Effects on Membranes Acetylcholine may participate directly in other neural membrane processes. It has been postulated (reviewed by Nachmansohn, 1970) that ACh molecules help to activate the Na^+ and K^+ permeabilities underlying the nerve impulse. The evidence is mainly indirect: nerve axons sometimes show anticholinesterase activity, and antagonists such as dTC sometimes interfere with axonal transmission.

Actions of ACh and cholinergic drugs on presynaptic membranes have been suggested for two systems: the myoneural junction (reviewed

by Riker and Okamoto, 1969) and sympathetic ganglionic transmission (reviewed by Burn, 1963). Investigators do not agree on the direction of this postulated presynaptic effect. Some experiments show that ACh enhances further ACh release (Koelle, 1962), while others show a marked depression (Ciani and Edwards, 1963; Hubbard and Yokota, 1964).

Structure - Activity Relations at the Acetylcholine Receptor

The Bis-quaternary Ammonium Compounds An early attempt to correlate structure with function of cholinergic drugs centered around the bis-quaternary ammonium compounds, introduced by Barlow and Ing (1948) and by Paton and collaborators (reviewed by Paton and Zaimis, 1952). These drugs have the general formula, $(CH_3)_3\overset{+}{N}(CH_2)_n\overset{+}{N}(CH_3)_3$. Decamethonium (n=10) is an agonist at the myoneural junction and thus blocks by desensitization. At autonomic ganglia, decamethonium has very little potency but pentamethonium (n=5) and hexamethonium (n=6) become very potent antagonists. However, the mechanism is now competitive inhibition of the receptor, similar to that caused by d-tubocurarine.

Paton and Zaimis (1952) asked whether these drugs could help to explain some molecular aspects of cholinergic transmission. Perhaps the activity peaks in the bis-onium series represent the distance between neighboring ACh receptors in the nicotinic or ganglionic postsynaptic membrane. However, the flexibility of these chains, and their relative lack of structure, prohibit us from drawing further conclusions. "The literature is full of shipwrecks in the quicksands of 'structure - action - relationship,' and it is obvious that our biophysical knowledge is inadequate to the pharmacological strains placed upon it." (Paton and Zaimis, 1952). We shall briefly summarize several recent attempts to explore this same "structure - action relationship."

Covalent Reactions at the Receptor Rang and his colleagues (Gill and Rang, 1966; Rang and Ritter, 1969) synthesized several decamethonium derivatives, including an irreversible alkylating agent (diphenyldecamethonium mustard). In the chick biventer cervicis and in leech muscle, these compounds seem to act with greater potency if they are applied at the same time as carbachol or succinylcholine. This potentiation has been termed the "metaphilic" effect, and it may arise from molecular changes in the receptor during desensitization.

Another approach, that of Karlin and his associates, started with the use of a two-step chemical procedure for modifying the receptor in Electrophorus electroplaques (Karlin and Bartels, 1966). A reduction with dithiothreitol, reversible by any of several oxidizing agents, was followed by irreversible alkylation with maleimide derivatives. Some maleimide derivatives caused inactivation of the receptors; others produced permanent depolarization (Karlin and Winnik, 1968; Silman and Karlin, 1969). From the structures involved, Karlin (1969) deduced that a negative subsite and a sulfhydryl group in the receptor decreased their separation from 12Å in the inactive state to 9Å in the active state. Karlin also employed a tritiated maleimide derivative (Karlin et al., 1970) as an affinity label for counting receptors. Unfortunately, the label seems only partially specific for the ACh receptor, because only partial protection occurs with cholinergic agents. Nevertheless, the "specific" fraction appears to involve about as many bound label molecules per electroplaque as there are acetylcholinesterase molecules.

Cooperative Interactions The concept of macromolecular systems as sites for regulatory and cooperative interactions has been applied to the Electrophorus acetylcholine receptor by Changeux and Podleski (1968). The underlying principle here is that the shape of the dose-response curve for depolarizing drugs gives a clue to the stoichiometry of binding and the mechanisms of activation. In some cases the addition of cholinergic activators changes the shape of the curve from

sigmoid to hyperbolic, indicating a significant but unknown change in the mechanisms of activation.

Conformational Analysis Another approach to the molecular nature of ACh receptors has been to analyze the conformation of known cholinergic drugs. The basic assumption here is that examination of a series of drugs--particularly those of rigid structure--with similar potency will reveal certain constancies in molecular structure; these necessary features presumably are complementary to binding sites on the receptor. Beers and Reich (1970) concluded that the nicotinic receptor exploits the quaternary ammonium moiety and the carbonyl oxygen; the muscarinic receptor, on the other hand, interacts with the quaternary ammonium, the ester oxygen, and the methyl group. Similar conformational analyses resulted in the estimation of a range of bond angles and lengths essential for nicotinic (Chothia and Pauling, 1970) and muscarinic (Baker et al., 1971) activity. In this connection it has been emphasized that acetylcholine's conformation in the crystal is only one of several energetically possible states.

These and other molecular aspects of drug-receptor interactions have been treated in several recent reviews and symposia (Danielli et al., 1970; Andersen and Jansen, 1970; Porter and O'Connor, 1970; Triggle et al., 1971).

In 1952, Paton and Zaimis found little basis for developing a theory to "account for the way in which a drug such as decamethonium or hexamethonium can select, from the protean manifestations of acetylcholine action, that of endplate stimulation or of ganglion competition alone." Perhaps the theories advanced since 1952 will help to accomplish this task. Its completion will bring the mechanistic dream of Helmholtz, Ludwig, Du Bois-Reymond, and Brücke one step closer to fruition.

Elapid and Hydropheid Snake Venom Neurotoxins May Bind Irreversibly to Acetylcholine Receptors

Elapid (cobras, mambas, sambas, corals) and hydropheid (sea snake) venoms contain a variety of proteins which act on cell membranes. These agents include cardiac muscle depolarizers, hemolytic factors, neurotoxins, anticholinesterases, and phospholipase A. However, it has been known for about a century that these snakes kill their prey primarily because the venom interferes with neuromuscular functions (see reviews by Meldrum, 1965b; Jiménez-Porras, 1968). This section shall summarize modern chemical and electrophysiological studies with the toxins responsible for this interference--the "curariform" toxins.

It is first worth noting briefly, however, that the cardiac muscle depolarizers and the hemolytic factors show close similarities, both in size and in amino-acid sequence, to the curariform toxin (D. Eaker, personal communication). We might therefore hope that an understanding of the curariform toxin's action will have some relevance to membrane processes in general.

It is also parenthetically interesting that Bungarus multicinctus, and possibly other snakes, produce an additional neuromuscular toxin. This agent apparently acts presynaptically by interfering with the ACh release mechanism (Chang and Lee, 1963; Lee and Chang, 1966), thus blocking normal transmission. Called β-bungarotoxin, the toxin molecule is several times larger than the curariform toxin in the same venom (Hamaguchi et al., 1968).

Chemical Properties The curariform toxins have a molecular weight of 6,000 to 8,000, depending on the species (see below); other physical properties show little variation among species (reviews by Jiménez-Porras, 1968; Lee, 1970). They are all strongly basic, having isoelectric points greater than 10, and are stable to heat treatments which destroy other toxin components of the venom. The toxins contain large amounts of cystine, the maintenance of whose disulfide bonds seems vital for toxicity. The toxins are readily water soluble.

Their behavior during analytical ultracentrifugation suggests that they are simple monomolecular spheres (Karlsson et al., 1966; Botes and Strydom, 1969), although dimers form under certain conditions (Karlsson et al., 1971).

Data now are available on the amino acid sequence of homologous toxins from different species. Two subclasses of toxins exist (Table I). One group consists of 61 or 62 amino-acid residues cross-linked by four disulfide bonds; the second group has 71 to 74 residues and five disulfide bonds. Within each group and between the two groups, the toxins show extensive similarities in their sequences. Fig. I-1 shows the sequence of toxin T_3 from Naja naja siamensis (kaouthia) venom; I used this toxin in my studies.

Lack of Three-Dimensional Information In their native conformation (disulfide bonds intact), the toxins are fairly resistant to the action of proteolytic enzymes. This fact has hindered attempts to locate the disulfide bonds and to gain other structural information. The clustering of basic amino-acid residues prompted the suggestion (Eaker and Porath, 1967; Yang et al., 1969) that positions 26 thru 40 of the 61-amino residue version contain the active site. However, when the sequence of the 71-residue Naja naja siamensis toxin was determined, this putative active site did not appear.

It seems clear that we must await a knowledge of the three-dimensional and disulfide bond structure in order to determine the stereochemistry of the toxin's action. The most promising route to this goal is probably X-ray crystallography.

Physiological Studies We may distinguish stages in the specificity of studies on elapid and hydropheid curariform toxins. In the first step, investigators began using defined fractions of venom to isolate the neuromuscular blocking component. The physiological assays usually consisted simply of noting that death came from respiratory failure, but in vitro studies have appeared. For example, Carey and Wright (1961) employed the rat phrenic nerve-

Table I

Curariform toxins from elapid and hydropheid snake venoms, grouped according to the number of amino-acid residues and disulfide bonds

Snake	Toxin (trivial name)	Reference
A. 61 or 62 amino-acid residues and 4 disulfide bonds		
Naja naja atra (Formosan cobra)	Cobrotoxin	Sasaki, 1957a,b Yang et al., 1969
Naja nigricollis (African spitting cobra, 'Mfesi)	Toxin α	Karlsson et al, 1966
Naja haje haje (Egyptian cobra)	Toxin α	Botes and Strydom, 1969
Bungarus fasciatus (Banded krait)	--	Tamiya et al., 1970
Hemachatus (Sepedon) haemachatus (Ringhals cobra)	(Peaks 3, 5)	Porath, 1966
Naja nivea (Cape cobra)	Toxins α, β	Botes, 1970
Laticauda semi-fasciata (sea snake)	Erabutoxins a,b,c	Tamiya and Arai, 1966
Laticauda laticaudata (sea snake)	Laticotoxin a	Tamiya et al., 1967
Laticauda colubrina (Banded sea snake)	Laticotoxin a	Sato et al., 1969
Enhydrina schistosa (common sea snake)	--	E. Karlsson, S. Kadin, D. Eaker, (in preparation)
B. 71 to 74 residues, 5 disulfide bonds		
Naja nivea (Cape cobra)	Toxin α	Botes, 1970
Naja naja siamensis (kaouthia) (Thailand cobra)	Toxin T	Karlsson et al., 1971
Naja naja naja (spectacled Indian cobra)	Toxins T_3, T_4	Karlsson et al., 1971
Bungarus multicinctus (Formosan banded krait)	α-bungarotoxin	K. Narita, C.Y. Lee (unpublished)

Naja naja siamensis toxin T3

1

H-ILE ARG CYS PHE ILE THR PRO ASP ILE THR SER LYS ASP CYS PRO ASN GLY HIS VAL CYS TYR THR LYS

THR TRP CYS ASP ALA PHE CYS SER ILE ARG GLY LYS ARG VAL ASP LEU GLY CYS ALA ALA THR CYS PRO THR VAL

71

LYS THR GLY VAL ASN ILE GLN CYS CYS SER THR ASP ASN CYS ASN PRO PHE PRO THR ARG LYS ARG PRO-OH

Figure I-1. The amino-acid sequence of Naja naja siamensis (kaouthia) toxin T_3 (D. Eaker and H. Arnberg, in preparation).

diaphragm preparation. Chang and Lee (1963) also used this preparation and the frog rectus abdominus, chick biventer cervicis nerve-muscle, and guinea-pig ileum. Tamiya and Arai (1966) employed the frog sciatic - sartorius and rectus abdominus preparations.

Protection by Cholinergic Agents Such studies with isolated organs produced an important piece of information. Dimethyltubocurarine, applied simultaneously with crude Bungarus multicinctus (Chang, 1960) or Naja naja atra (Su, 1960) venom, protects the frog rectus abdominus muscle against the venom's effects. A similar protection was observed against the pure toxins in the chick biventer cervicis muscle (Lee and Chang, 1966; Su et al., 1967). Since curarine binds to the ACh receptor (Jenkinson, 1960), this suggests that the venoms also exert their action by such binding. Chapter V deals with extensions of these observations.

Electrophysiological Studies A second stage of specificity was attained with the recording of postsynaptic depolarization rather than muscle tension (Peng, 1960; Meldrum, 1965a; Change and Lee, 1966). These experiments confirmed that isolated polypeptide components of elapid venoms block acetylcholine's depolarizing action at the skeletal myoneural junction. Chapter III treats this action in greater detail.

Autoradiography with ^{131}I Toxin In addition to the protection experiments with curare, another line of evidence also suggests that elapid toxins bind directly and irreversibly to the ACh receptor. The pure curariform toxins from Bungarus multicinctus (Lee and Tseng, 1966), Naja naja (Tseng et al., 1968), and Laticauda semifasciata (Sato et al., 1970) were labeled with ^{131}I; mice were killed by injections of the labeled toxin. Autoradiography of the diaphragm showed that the label remained only at the endplate region. When rats received injections of labeled bungarotoxin 60 days after severance of the phrenic nerve, the label spread out over the whole length of the muscle fibers (Lee et al., 1967). This distribution of

label conforms to the spread of receptors after denervation demonstrated electrophysiologically (Thesleff, 1960; Miledi, 1963) and autoradiographically with ^{14}C-curarine (Waser and Lüthi, 1957; Waser, 1960).

Choice of Preparations

The preceding section reviews the evidence that cobra toxin blocks the vertebrate myoneural junction. In my experiments, I sought to locate this action at the ACh receptors themselves and to determine which receptors are susceptible to the toxin.

The working hypothesis, that only nicotinic receptors would show sensitivity, arises from two lines of evidence. First, there are no reports that the toxin blocks noncholinergic transmission.* Second, α-bungarotoxin fails to block muscarinic transmission in the guinea pig ileum (Chang and Lee, 1963).

With this evidence as a background, I chose at least one synapse from each of three groups: nicotinic, muscarinic, and molluscan (not assignable to either category, see p. 5).

Frog Myoneural Junction This preparation has interested scientists since the time of Volta and Galvani. Claude Bernard used it to localize the site of curare action; Helmholtz and Adrian chose it to investigate impulse conduction.

More recently, frog muscles were employed by Ling and Gerard (1949), Nastuk and Hodgkin (1950), Fatt and Katz (1951), Nastuk (1953), and del Castillo and Katz (1955), who pioneered the use of glass-

*Parnas and Russell (1967) found that crude elapid venoms interfered with crustacean neuromuscular transmission. However, these effects were reversible with low venom concentrations and probably arose from nerve blockage, which was caused by toxins different from the curariform component.

tipped microelectrodes for intracellular recording and for micro-application of drugs.

The frog myoneural junction now offers the best opportunity to study the biophysics and pharmacology of nicotinic transmission. Hence, I employed this preparation for most of my experiments. My biophysical techniques represent only minor improvements on the methods of the authors cited above, although advances in electronic technology have provided instruments of greater stability and computer technology has enhanced the capabilities of on-line and off-line processing systems (Methods).

Skate Electroplaque The tails of all skates (Raja) contain weak electric organs of unknown function. These organs are embryonically related to muscle. It is therefore not surprising that synaptic transmission to electroplaques shares many characteristics with neuromuscular transmission. These features include nicotinic pharmacology, as shown by sensitivity to dTC (Brock and Eccles, 1952). Postsynaptic potentials (PSPs) are increased by anticholinesterase (Grundfest and Bennett, 1961). The reversal potential for the postsynaptic current is about equal to that for the endplate current. Furthermore, spontaneous miniature PSPs and quantization of the PSP have been noted, and the quantitative Ca^{++} dependence of ACh release is similar to that at the myoneural junction (Lester, 1970c). In view of these similarities, I thought it worthwhile to compare cobra toxin's action in this preparation with its effect on neuromuscular transmission.

Torpedo nobiliana Electroplaques The electroplaques of the giant electric ray, Torpedo nobiliana, are classically known to exhibit nicotinic pharmacology, as shown by sensitivity to curare.

They provide a less convenient electrophysiological preparation than Raja electroplaques. The cells are large, thin discs, in which intracellular penetrations are difficult to maintain and whose cable properties do not allow PSPs to sum for the entire cell. Furthermore,

pharmacological investigations are difficult because bath-applied agents fail to penetrate past the surface cells (see Chapter III).

Nevertheless, Torpedo electric organs contain so much ACh receptor material that they may provide suitable material for biochemical studies. For this reason, I considered it important to explore their response to cobra toxin.

Aplysia californica Abdominal Ganglion The recognition and permeation functions at Aplysia and other molluscan ACh receptors have been reviewed (pp. 4-6).

I chose an easily studied synapse in the abdominal ganglion. The presynaptic fiber runs in the right mantle connective; the parabolic burster (Strumwasser, 1965), also called R15 (Frazier et al., 1967), is the postsynaptic cell. This synapse is cholinergic (F. Strumwasser, personal communication; see also Chapter III).

Isolated Frog Heart The isolated frog heart has been used to investigate chemical transmission ever since Loewi's studies (1921) first established that the process occurs. The receptors are muscarinic according to several criteria (see, for instance, Goodman and Gilman, 1965).

The heart is also interesting because its ACh receptors produce inhibitory K^+ currents. Therefore, we can learn whether the toxin's specificity follows the permeation rather than the recognition function, by comparing this preparation with the excitatory muscarinic receptors of the guinea pig ileum (Chang and Lee, 1963). The toxin's specificity for the permeation function cannot be studied with nicotinic receptors, because there are no known inhibitory nicotinic receptors.

CHAPTER II

METHODS

Frog Sartorius Myoneural Junction

Solutions For my experiments, a Ringer solution bathed the muscle. The standard Ringer contained NaCl, 112 mM; KCl, 2.5 mM; $MgCl_2$, 4 mM; $CaCl_2$, 2 mM; and Tris, 5 mM (pH 7.3). It was often desirable to decrease the quantal content to just the point where no endplate potential (EPP) exceeded spike threshold in any fiber. I accomplished this, unless otherwise stated, by decreasing $CaCl_2$ to 0.5 - 0.7 mM, with isotonic substitution of NaCl (del Castillo and Katz, 1954a).

The experiments were performed with toxin T_3 from the venom of Naja naja siamensis - (kaouthia) (Thailand cobra), kindly supplied as the crystalline powder by D. Eaker (Karlsson et al., 1971). The toxin was stored at 4^{o} in a stock concentration of about 1 mg/ml in acetate buffer, 50 mM, pH 5.0. Concentrations were checked by measuring absorption at 280 mμ against a known absorbance value (1 cm path length, 1 mg/ml) of 1.05 (D. Eaker, personal communication). The toxin solutions were diluted to stated concentrations (1.6×10^{-7} M, or less) in the appropriate Ringer just before use. d-Tubocurarine chloride was obtained from K & K Laboratories, Inc.; all other organic chemicals were supplied by Sigma.

Experimental Chamber The experimental chamber held 3 - 5 ml of solution, depending on the fluid level. For solution changes, a dead volume of 2 - 3 ml was introduced by the connecting tubes and bubble traps. Studies on dye dilution showed that a solution change was 50% complete after 10 ml, 95% after 25 ml, and more than 99% after 50 ml had flowed through the system. Flow rates as high as 10 to 15 ml/min could be employed, giving essentially complete changes within 5 minutes.

Recording Recording was accomplished with glass microelectrodes filled by boiling with 3 M KCl. Tip resistances ranged from 5 to 30 MΩ. The low-resistance pipettes were preferred for single preparations involving measurements on miniature endplate potentials (MEPPs) (Chapter III). For repeated penetrations in the same fiber over a period of several hours, greater recording noise with the high-resistance pipettes was accepted in return for the decreased probability of damage to the muscle fiber membrane.

Transmembrane potentials were recorded between the microelectrode and an Ag-AgCl indifferent electrode located in the bath. The recording amplifier (designed by J.P. Hervey) has been described elsewhere (Purple, 1964). It consisted of an augmented follower with bucking and polarizing circuitry plus an active bridge for measuring resistance.

Micro-ionophoresis Glass micropipette electrodes were also used for focal ionophoretic delivery of drugs. Since my experiments often required the same ionophoretic dose over a time course of 1 - 2 hours (see Chapter V), several precautions insured that the ionophoretic pipette did not change its efficacy due to mechanical drift, slow desensitization (del Castillo and Katz, 1955), or change in the effective amount of drug delivered per current pulse. I monitored the ACh or Carb potentials over an initial control period of 15 - 30 minutes; only preparations giving stable responses were used. Also, the ACh or Carb potentials recovered within 15 minutes after relatively strong desensitizing treatments which I initially feared might change the characteristics of the drug pipette.

Multiple Penetrations in Experiments with Bath-Applied Drugs For protection experiments with bath-applied drugs (Chapter V), the goal was to overcome the variations among individual fibers (see Results) by averaging over the results for several fibers in a single muscle. The external recording method of Fatt (1950) was inappropriate because in preliminary experiments the cobra toxin failed to block

transmission to the fibers on the interior of the muscle. This presumably occurred because the toxin did not readily penetrate past the superficial fibers. I therefore chose to monitor the EPP in about five identified superficial fibers over the complete experiment (often as long as 8 hours). This was accomplished by sketching a portion of the muscle as it appeared under the stereomicroscope; fibers were then impaled sequentially for measurements at various stages in the experiment. The technique has been described by Fatt and Katz (1950).

Computerized Data Collection and Analysis

In my experiments, it was of prime importance to monitor continuously the degree of block produced by toxin or by reversible cholinergic agents. For this purpose, two factors dictated the use of automatic averaging rather than simply inspecting oscilloscope traces. Firstly, fluctuations among successive responses were caused by the quantal mechanism of ACh release (del Castillo and Katz, 1945a). Secondly, when the treatments blocked most of the ACh receptors, EPP amplitudes often were comparable to or smaller than the electrical noise in the recording instruments. An additional requirement arose from the need to measure many individual EPPs and MEPPs for the quantal content determination (Chapter III).

On-Line Analysis: CAT An on-line digital computer provided the necessary signal-processing capabilities (see Fig. II-1). One goal of the CAT ("Computer of Average Transients") system was to allow the experimenter to control the experiment without leaving the electrically shielded room where the recording took place. In addition to the electrophysiological equipment, the shielded room contained timing and stimulating equipment, a digital-to-analog output scope which displayed averaged waveforms and protocol information, and a bank of switches for selecting among program options. The timing was synchronized to a master sampling clock (usually 10 kHz) located in the data interface.

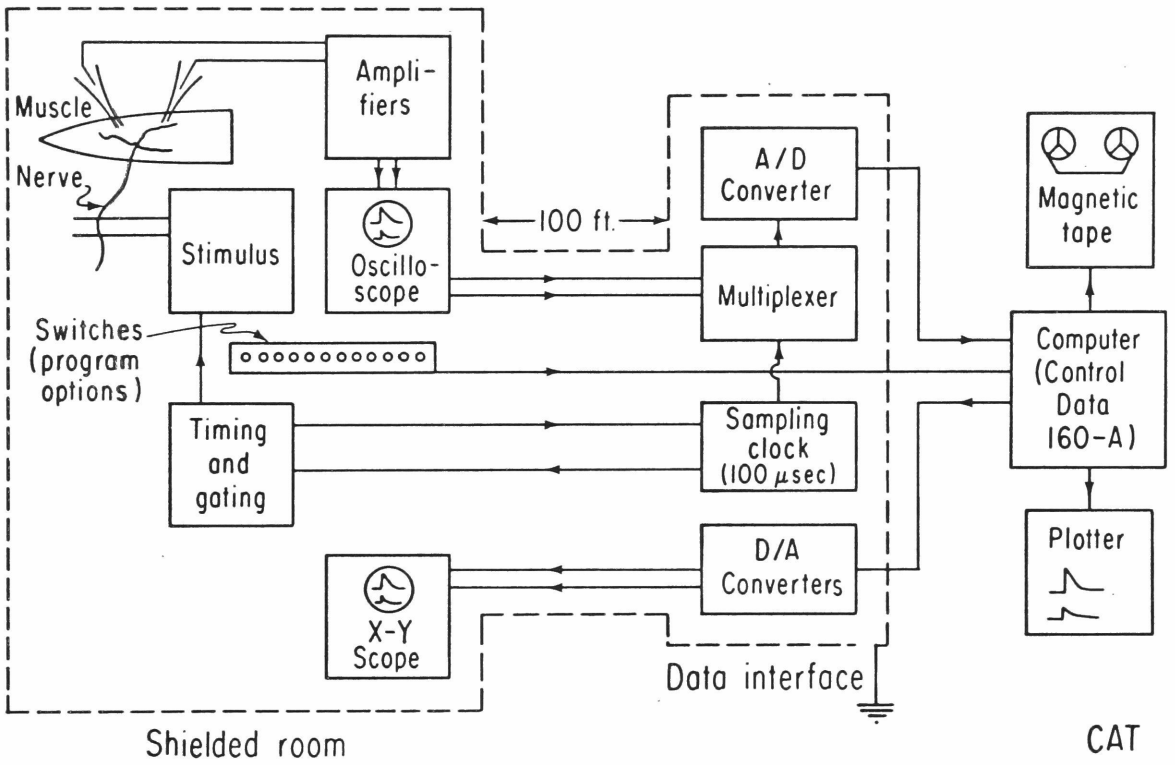


Figure II-1. Equipment configuration for experiments with the frog sartorius myoneural junction. Two intracellular microelectrodes are shown supplying signals for two data channels.

The signal path led from the electrodes and cathode followers to the vertical signal amplifiers of an oscilloscope (Tektronix 502A). Shielded cables carried the signals to analog-to-digital converters in the data interference. A buffer received a single sweep of 1000 points (one channel, or more usually two 500-point channels). This sweep could be written on digital tape for further analysis by the RECAT system (see below). The CAT program added each sweep to a thousand-point array of running sums in core storage. A new average was then formed by dividing each point by the number of sweeps completed. A second buffer transmitted the averaged numbers to digital-to-analog converters in the data interface; the averaged sweep appeared on the display scope. At the end of each averaging series, the computer displayed the final average, produced a plot of the average, and wrote the sums and averages on tape.

Two data channels were available. These usually carried intracellular potential records from two different endplates, but in some experiments, one channel contained information on extracellular potential or on current through an ionophoretic pipette.

The CAT system also could accept data which had been recorded on analog tape with the proper timing signals. This arrangement was used for analyzing simultaneous presynaptic and postsynaptic intracellular potential records from the squid giant synapse (Lester, 1970a).

Off-Line Analysis: RECAT The CAT output tape provided the input for the RECAT analysis (Fig. II-2) (actually, RECAT is an addition to the CAT program). With human help, RECAT transformed a series of single sweep records to an array of EPP or MEPP amplitudes on FORTRAN-compatible tape. Each sweep was read in from magnetic tape and appeared on the display scope, accompanied by two horizontal "pointer" lines. The positions of these pointers were continually controlled by two potentiometers which fed into analog-to-digital converters. One pointer was set to the baseline, the other to the peak; the computer read the difference and performed all necessary protocol checks.

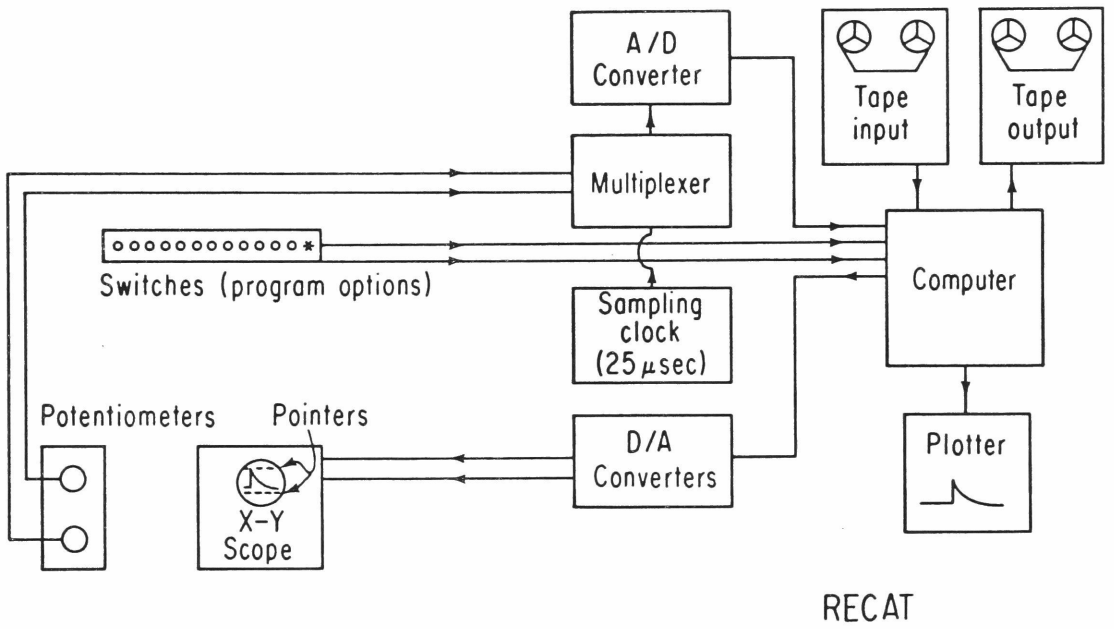


Figure II-2. The RECAT system for analyzing EPP and MEPP data. As explained in the text, the potentiometers control the positions of the pointers on the display scope. The computer used these positions to calculate response amplitudes.

With practice, I measured records at the rate of about one sweep every five seconds. A total of 6×10^4 sweeps were processed in this fashion. A totally automatic measurement of EPP amplitudes has since been developed utilizing new features of the computer's FORTRAN compiler (work of Norman Milkman).

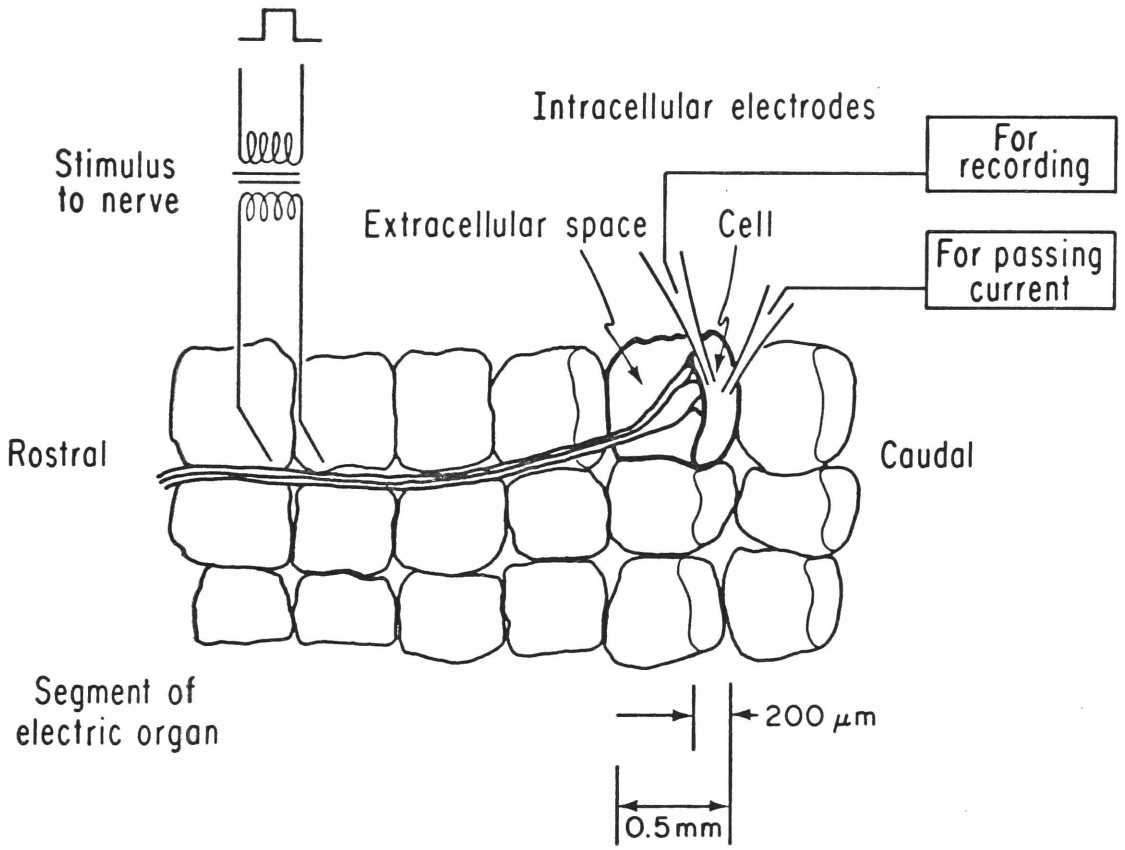
Skate Electroplaque Preparation

I used two skate species, Raja erinicea and Raja ocellata, which are commonly available at Woods Hole. Intracellular recording from skate electroplaques was first reported by Brock, Eccles, and Keynes (1953) and in detail by Brock and Eccles (1958). My procedures closely followed those of Grundfest and Bennett (1961). Segments of electric organ about 1 cm in length were used for these studies, as shown in Fig. II-3.

Solutions The experimental chamber (Lester, 1970a) had a volume of 0.5 ml and its temperature, usually about 15°, could be controlled within $\pm 0.25^{\circ}$. The chamber was continuously replenished by fresh solution, at a rate of about 1 ml/min. The elasmobranch Ringer solution (Brock and Eccles, 1958) had the following composition (mM): NaCl, 250; KCl, 5; $CaCl_2$, 4; $MgCl_2$, 2; urea, 333.3; Na phosphate buffer, 1.5 (pH 7.4).

Stimulating and Recording Paired silver wires, insulated except at the ends and mounted on a micromanipulator, served as stimulating electrodes. These were placed near nerve fibers at the rostral end of the preparation and intracellular records were taken from electroplaques near the caudal end. This arrangement gave the largest possible distance between the stimulating and recording electrodes, thereby minimizing electrical pickup in the recording circuit.

Recording could generally be accomplished with electrodes of about 5 MΩ tip resistance; the electroplaques were large and easy to impale. Signals were displayed on a storage oscilloscope and



Skate electroplaque preparation

Figure II-3. Intracellular recording from skate electroplaques, as described in text. Each electroplaque receives innervation from about three presynaptic fibers (Brock and Eccles, 1957); in favorable preparations, each input could be isolated by varying the stimulus parameters. The test electroplaque is shown penetrated by two microelectrodes for resistance studies (Lester, 1970c).

photographed with a Polaroid camera.

Other techniques duplicated those described for the frog myoneural junction.

Torpedo nobiliana Electroplaque Preparation

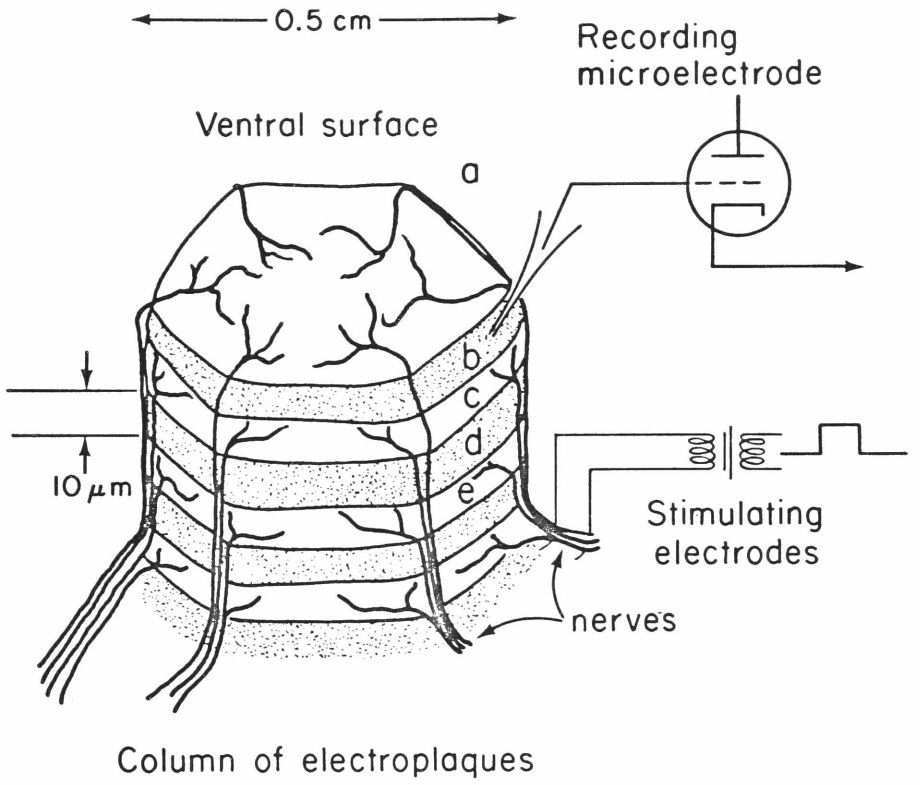
In Torpedo, the kidney-shaped electric organs form almost half the mass of each wing. Each organ contains several hundred columns of cells; the columns themselves consist of about a thousand stacked electroplaques.

Figure II-4 describes the techniques for stimulating the presynaptic nerve and intracellular recording from individual electroplaques (Bennett et al., 1961). These studies employed the frog muscle chamber (see p. 19). Solutions and all other procedures duplicated those described for skate electroplaques.

Aplysia californica Abdominal Ganglion

Experiments on the isolated abdominal ganglion of Aplysia californica, the sea hare, were conducted at room temperature in the chamber used for studies on frog muscles (see p. 19). The ganglion was bathed in artifical sea water. The right mantle connective nerve was led off into the moist stimulating chamber which ordinarily housed the frog motor nerve (Fig. II-5).

A tough connective tissue capsule encases the ganglion. Attempts to soften this sheath with pronase resulted in damage to synaptic transmission. Penetrations could, however, be successfully accomplished through the sheath if the microelectrodes (tip resistance 5 - 19 MΩ) were mounted on a micromanipulator with axial drive instead of the usual oblique approach. This procedure gave stable intracellular recordings for many hours.



Torpedo nobiliana electroplaque preparation

Figure II-4. Microelectrode recording from a column of Torpedo electroplaques. The ventral end of the column is shown. The electrode tip could be positioned above the column (a), within (b,d), or between (c,e) cells. The indifferent electrode (not shown) remained stationary in the bath. The stimulating current actually excited several presynaptic nerve trunks.

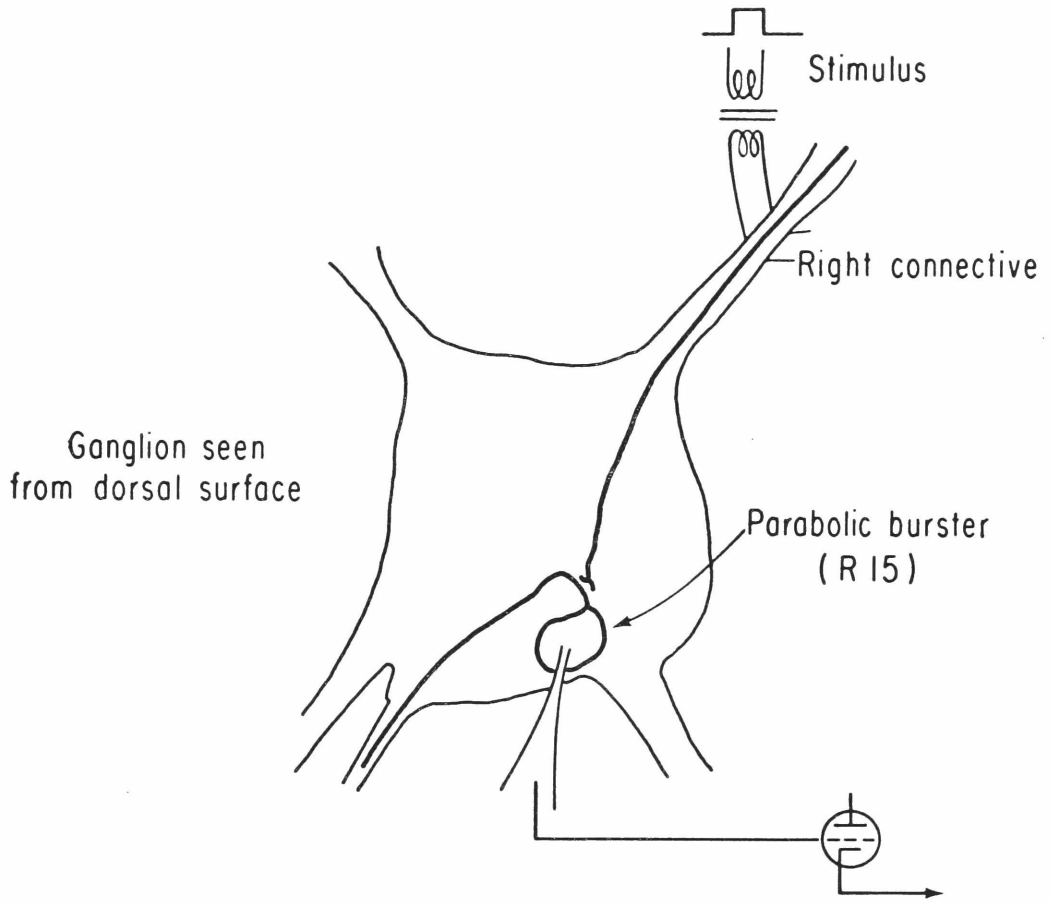


Figure II-5. Intracellular recording from an identified cell in the Aplysia abdominal ganglion.

Like all synapses in Aplysia ganglia, the axo-axonic junction is some electrical distance from the cell body, which is the site of recording. However, this PSP has a greater amplitude and faster rising phase than most others in R15 and in other Aplysia neurons. The actual synapse is probably less than one cable length from the recording electrode.

Isolated Frog Heart

For my experiments, the heart was pinned to a layer of silicone rubber (General Electric RTV-615) in the bottom of a dish at room temperature. Frog Ringer solution (see p. 19) was used.

Ten ml of solution was present in the dish and just covered the heart. Drugs were added as noted. Some of the experiments were performed in the presence of dTC, 3×10^{-5} w/v, in order to eliminate any effects of the toxin on parasympathetic ganglion cells in the heart. No differences were noted with and without dTC.

In the absence of drug treatments, the isolated heart beat steadily for 1-2 hours. Beating frequency was measured by recording the time required for 10 beats. In addition, I noted the relative strength of beats. The preparation responded within a few seconds to solution changes.

CHAPTER III

COBRA TOXIN BLOCKS NICOTINIC ACETYLCHOLINE RECEPTORS

Experiments with the Frog Myoneural Junction

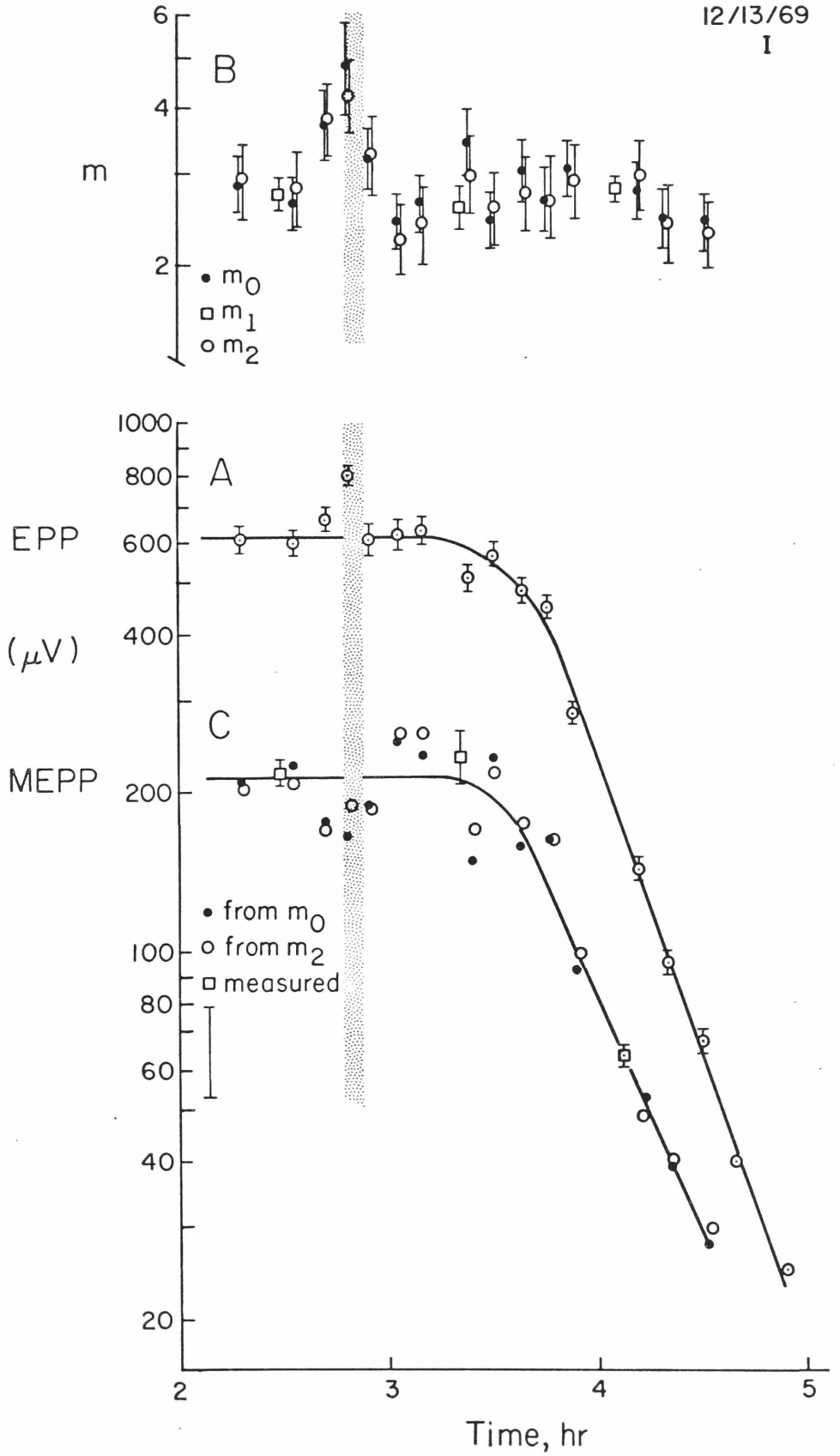
My initial investigations with the frog myoneural junction confined the cobra toxin's action to one locus: the postsynaptic ACh receptor. Three lines of evidence contributed to this conclusion: 1) quantal content analyses on EPPs; 2) experiments with micro-iontophoretic application of ACh; 3) comparisons of EPP time course before and after toxin application.

Quantal Content For extensive analyses on endplates treated with cobra toxin, I utilized a toxin concentration of 1.6×10^{-8} M with $[Ca^{++}] = 0.4$ mM (see Methods, Chapter II). The quantal content, m, ranged from 1.5 to 15 but usually was near three. This value lies in the range where three independent estimators of m may reliably be employed (Martin, 1966): m_o, from the number of failures; m_1, from the ratio of the average EPP to the mean MEPP amplitudes; and m_2, from the coefficient of variation of the EPP amplitude distribution. To minimize errors caused by trends, the determination of m_2 utilized a grouped linear regression analysis similar to that described by Colomo and Rahamimoff (1968).

Figure III-1 shows results of an experiment. After a delay, the toxin causes a decline in EPP amplitude. This decrease eventually attains an exponential rate. A simple explanation for this finding is that the toxin irreversibly inactivates a given fraction of the remaining ACh receptors per unit time (mechanisms of toxin action are treated in Chapter IV). Indeed, washing the preparation (not shown; see Fig. IV-1) caused only slight recovery after several hours.

In Figure III-1, the curves for EPP (A) and MEPP (C) amplitude remain parallel on semilogarithmic co-ordinates, while the curve B (showing the quantal content, m) remains essentially constant. These

Figure III-1. Postsynaptic action of cobra toxin on myoneural transmission. At cross-hatching chamber was flushed with solution containing toxin. ◐, ● and O, Measurements on a series of 128 EPPs. □, Measurements on spontaneously occurring miniature EPPs. A, Average EPP amplitude ± s.e. EPPs without error flags at later times were too small, relative to the recording noise, for accurate measurements on individual responses. B, Quantal content m ± s.e; (O) m_0, computed from the number of failures; (●) m_2, computed from the coefficient of variation of the EPP amplitude distribution; (□) m_1, computed by dividing EPP by miniature EPP amplitudes. C, Miniature EPP amplitude; □, measured directly. O, computed as EPP/m_0; ●, computed as EPP/m_2. The vertical line at the lower left gives approximate error limits for computed miniature EPPs.


12/13/69
I
B
m
6
4
2
• m_0
□ m_1
○ m_2
A
EPP
(μV)
1000
800
600
400
C
MEPP
200
100
80
60
40
20
• from m_0
○ from m_2
□ measured
2
3
4
5
Time, hr

observations support the interpretation that the toxin decreases the individual quantal size while not disturbing m, the number of quanta released.

Non-uniform Action on Different Regions of the Endplate Most experiments showed a decline in m as well as a diminution in MEPP amplitude. Figure III-2 illustrates such results. Results from fifteen endplates were averaged by comparing the changes in m and in MEPP amplitude when the EPP had dropped to about one-fourth its original amplitude. The averages (as a fraction of the value before treatment) were EPP amplitude, .26; MEPP amplitude, .41; m, .67.

Taken at face value, these data indicate an additional presynaptic action of the toxin on ACh release. Such effects have been proposed for several other agents which act postsynaptically (see Chapter I). In this case, however, the correct explanation seems to arise from non-uniform postsynaptic action of the toxin on different regions of the endplate, which can often attain lengths of 1 mm (R. Miledi, unpublished results). This complication can naturally arise from local variations in the accessibility of the endplate to toxin molecules, caused by variations in the connective tissue and Schwann cell covering (Birks et al., 1960). Also, some branches of the endplate may lie in the cleft between adjacent fibers, while others lie on the more exposed superficial surface (Kühne, 1887; Couteaux, 1947; Cole, 1955). (A detailed treatment of diffusion barriers and binding sides for the toxin, and their contribution to variability and latency, is given in Chapter IV.)

The following observations suggest that such positional differences account for sizeable variations in the time course of toxin action. Firstly, variations as great as a factor of 3 occurred in the delay before toxin action and in the inactivation rate constant (slope of curve A in Fig. III-1; see Chapter IV) even when different endplates a few hundred μm apart were monitored simultaneously in the same muscle. Secondly, toxin treatments which caused decreases by a

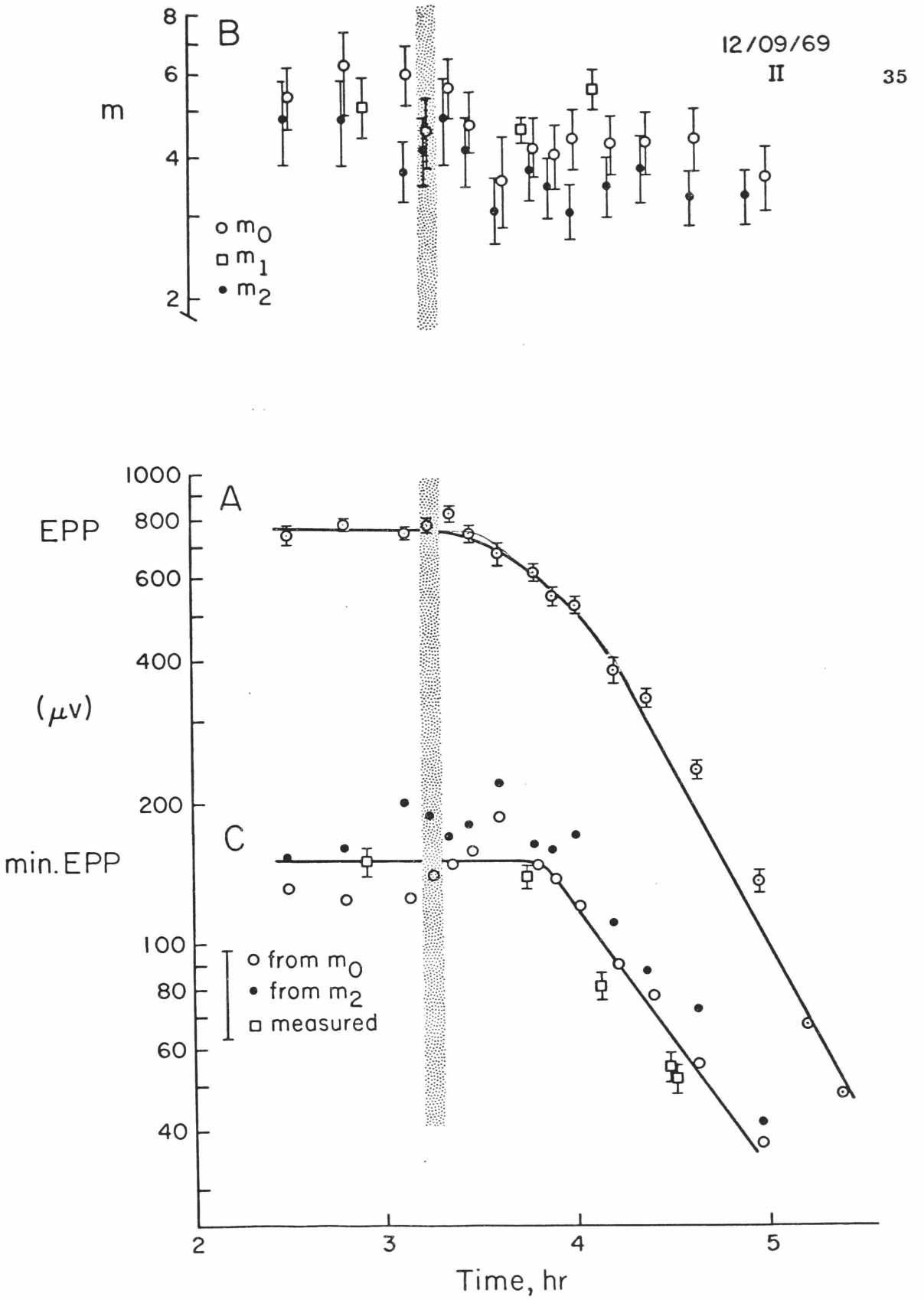


Figure III-2. Data from another endplate, in which m appeared to decline as well as MEPP amplitude. See Figure III-1 for legend; text for discussion.

factor of 100 in superficial EPPs, still allowed endplates on deep fibers to develop EPPs larger than impuse threshold. (Also noted by Lee and Chang, 1966.)

If the toxin acts non-uniformly along an endplate, then the coefficient of variation of the MEPP amplitude distribution should increase. Recording noise made it somewhat unreliable to measure this effect. There was, however, a significant increase, from .23 during the control period to .36 when the toxin had decreased the EPP by at least a factor of two (fourteen endplates). This broadening tends to lower all three estimates of m. The number of failures, which determines m_o, is overestimated because responses consisting of one small MEPP are treated as failures. The average MEPP amplitude is likewise underestimated as small MEPPs go undetected, thus decreasing m_1. Finally, the broadened MEPP distribution leads to a broader EPP amplitude distribution, decreasing m_2.

Higher Toxin Concentrations Figure III-3 shows data taken from another experiment, in 0.6 mM Ca^{++}. Here m_2 was approximately 15 (min. EPPs were not measured so m_1 was unknown; m_o is unavailable in this range, because no failures occur). Toxin concentration was 1.14×10^{-7} M.

Under these conditions, the behavior of the EPP amplitude fluctuations still supports the hypothesis that the toxin acts irreversibly on the quantal size but not on the quantal content. With this higher toxin concentration, the delay time is short and the action more rapid. (For a discussion of the quantitative dose-response studies, see Chapter IV.) The average (heavy line) and two standard deviations (lighter lines) have been marked for the first 80 points before the toxin begins to act (segment A to B). An exponential decline was fitted to the 120 EPPs between C and D. Assuming that the toxin affected only the scale of the distribution, the same fractional limits have been continued to the exponential decline portion. Parallel lines result on the semilogarithmic plot. In both segments (A-B and C-D), 5% of the EPPs lie outside the limits.

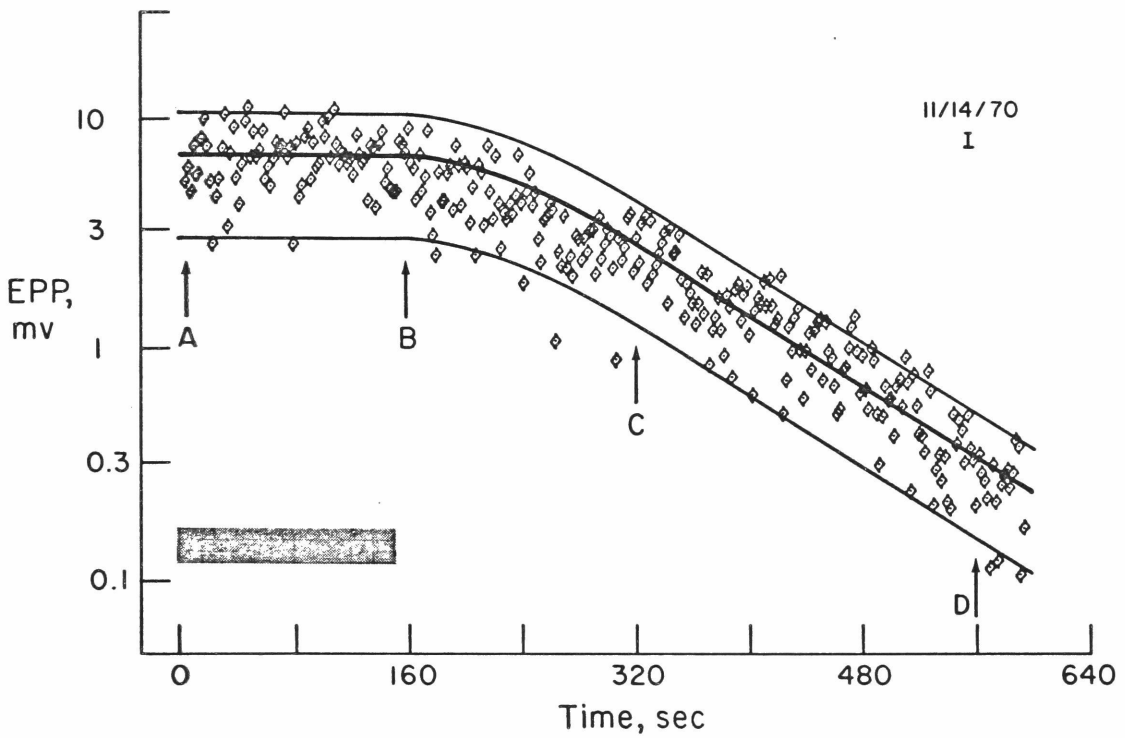


Figure III-3. EPP amplitude during treatment with toxin at a concentration of 1.14×10^{-7} M. Shaded area represents the time required to flush the experimental chamber with the toxin solution. EPP amplitudes greater than 1 mv have been corrected for nonlinear summation as described by Martin (1955) (see Chapter IV).

Therefore, the EPP amplitude distribution has not become broader, relative to the mean, than it was before toxin treatment. A decrease in m, however, would have increased the breadth. For instance, the Poisson formula predicts that, if m decreased by 40%, then 12% of the EPPs would fall outside of the limits on segment C-D. As found in the experiments with lower toxin concentration, the toxin seems to act only by decreasing MEPP amplitude.

Micro-ionophoretic Application of Acetylcholine The results described above prove that cobra toxin decreases the quantal size of ACh action at the myoneural junction. This diminution could arise from two causes: 1) presynaptically, decreased ACh content per quantum; 2) postsynaptically, decreased ACh sensitivity of the muscle fiber membrane.

Figure III-4 shows that possibility (2) is the case. ACh release by the nerve depends on the presence of Ca^{++}, but the response to micro-ionophoretically applied ACh remains substantially unchanged when Ca^{++} is removed (trace B). The toxin, however, blocks both the ACh potential and the EPP (D-F).

Note that Figure III-4f seems to indicate that the ACh potential recovers slightly with washing. It is extremely difficult to evaluate such small changes. One might suppose that any reversibility of the toxin's action would show up more clearly with micro-ionophoretic application, because the receptors accessible to the externally applied ACh might also be washed more thoroughly (Goldsmith, 1963). However, mechanical drifts between the muscle fiber and the ACh pipette can also cause changes of this magnitude. If any reversibility exists, it awaits study with more appropriate techniques.

Time Course of the EPP We may eliminate other effects of the toxin by comparing the time courses of averaged EPP waveforms before and after treatment (Fig. III-5). Although the absolute values of the EPPs differ by a factor of 80, the two traces superimpose after magnification to the same peak amplitude.

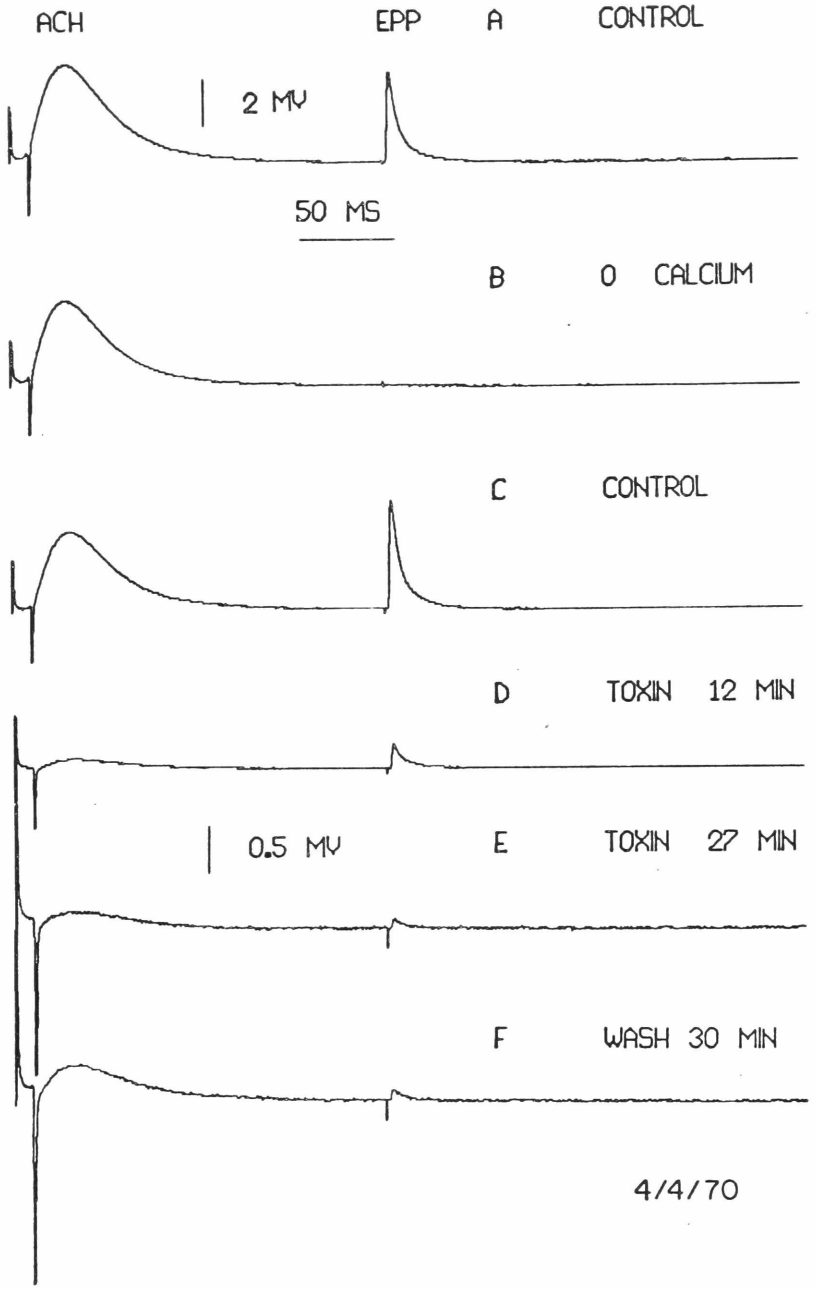


Figure III-4. Effect of cobra toxin on the response to micro-iontophoretically applied ACh. Control solution contained 0.6 mM Ca^{++}; toxin solution also contained 5 x 10^{-8} M toxin. ACh was applied with a current of 15 nA for 10 msec. Brief capacitative transients mark the beginning and end of the ACh pulse. Each trace represents the average of 16 sweeps. Vertical calibration: A-D, 2 mv; E, F, 0.5 mv.

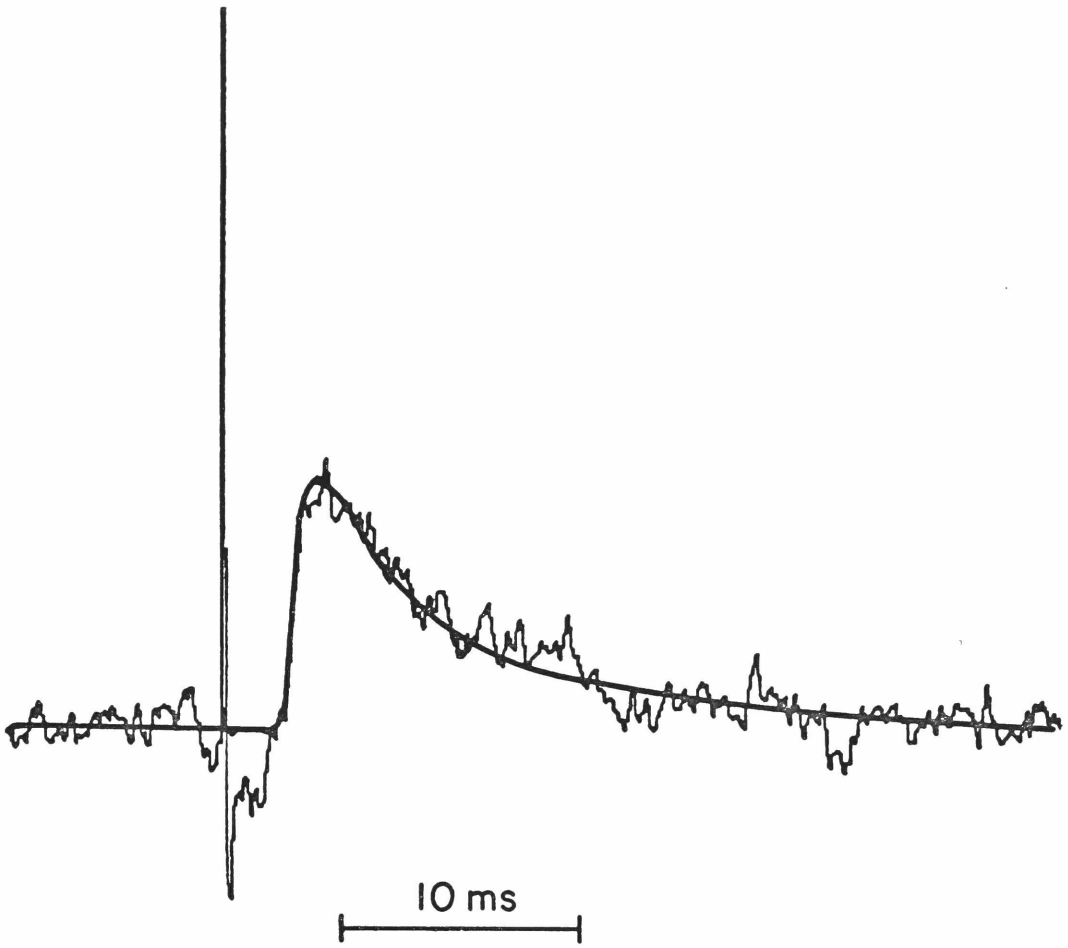


Figure III-5. Waveforms of 128 averaged EPPs, before (smooth, heavy trace) and 150 min after (light, noisy trace) treatment with cobra toxin as described in the text. Waveforms have been amplified to match peak amplitudes; actual amplitudes were 2.1 mV before, 25 μV after. Amplifier was capacitance-coupled with a time constant of 100 ms.

Anticholinesterases such as prostigmine prolong both the rise and fall of the EPP (Fatt and Katz, 1951). Therefore, the toxin appears not to affect the acetylcholinesterase. Also, the latency between the stimulus artifact and the start of the EPP remains unaffected; therefore, propagation of the presynaptic impulse and synaptic delay (Katz and Miledi, 1965a, b; Lester, 1970a) do not change significantly. Chang and Lee (1966) provided valuable evidence on this point. They recorded the impulse at the nerve terminal and found that it remained unchanged during treatment with cobra toxin, while the endplate current declined. The rising phase of the EPP appears constant, so the kinetics of ACh release have not altered (Katz and Miledi, 1965 b, c). The constancy of the falling phase indicates that the passive resistance and capacitance of the muscle fiber also remain the same.

In three out of 16 endplates, the EPP after toxin treatment had a significantly faster falling phase than during the control period. In one of these cases, the resting potential had declined by 20 mv and the difference was ascribed to a decreased membrane resistance; the experiment was discarded. For the other two cases, examination of the individual records showed that another active endplate, several cable lengths away, was contributing to the signal during the control period. The toxin apparently inactivated the distant endplate more quickly than the near one in both cases.

Absence of Effects on Other Processes The resting potential showed no significant changes for any of the toxin treatments used. Furthermore, in 13 endplates, when the EPP had dropped to less than half its control value, the frequency of MEPPs averaged .97 times the control (range .48 to 1.53). Chang and Lee (1966) have observed no changes in the muscle fiber action potential after toxin treatment.

The foregoing observations all lead to the conclusion that the cobra toxin acts at the frog myoneural junction solely on ACh sensitivity.

Skate Electroplaque

Cobra toxin blocked cholinergic sensitivity in the skate electroplaque. Figure III-6 shows that both postsynaptic potentials (PSPs) and steady depolarizations, caused by bath-applied carbachol (Carb), decreased after toxin treatment. Like the EPP at the frog myoneural junction, the PSP declined exponentially with time, and no substantial recovery occurred after several hours' washing with fresh solution.

Block of Miniature Postsynaptic Potentials The toxin also blocked spontaneous miniature postsynaptic potentials (Lester, 1970c), which are analogous to MEPPs at the myoneural junction. The skate electroplaque's qualitative response to the toxin thus matches the results of the more extensive study of the endplate.

Toxin's Potency The toxin's potency, as defined by the inactivation rate constant (see Chapter IV), was about 15 times less in the skate preparation than in the frog myoneural junction. This potency did not vary strongly with temperature in the range 11° - 30° but the toxin showed much less activity below 11° (see Chapter IV).

Torpedo nobiliana Electroplaque

Figure III-7 shows that the toxin blocked PSPs in surface electroplaques from Torpedo nobiliana electric organs. As in the skate, there was little recovery with washing.

Lack of Penetration to Deep Electroplaques Figure III-8, from another preparation, shows that the toxin fails to penetrate past the surface electroplaque. Negative-going traces recorded at locations a and b (see Methods, Chapter II) indicate that postsynaptic currents are still flowing into subsurface electroplaques; and intracellular records, such as at d, confirm that these electroplaques are still active. At earlier times (see 17 minutes) diphasic waveforms appear in the surface electroplaque. I ascribe these waveforms to differences in latency between the weak PSP in the surface electroplaque

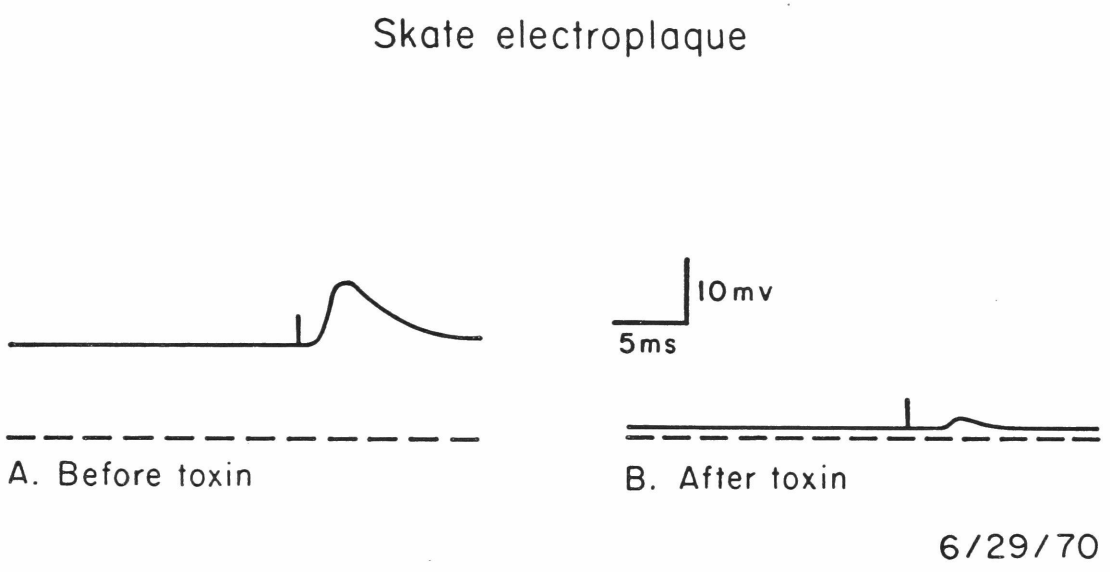


Figure III-6. Tracings of the skate electroplaque's resting potential and PSP (stimulus artifact has been retouched). Dashed line shows normal resting potential (90 mv). A few minutes prior to each measurement, Carb (3.3 μM) was added to the Ringer and caused a steady depolarization. Trace B was taken after a 60 minute exposure to a toxin concentration of 1.14×10^{-7} M.

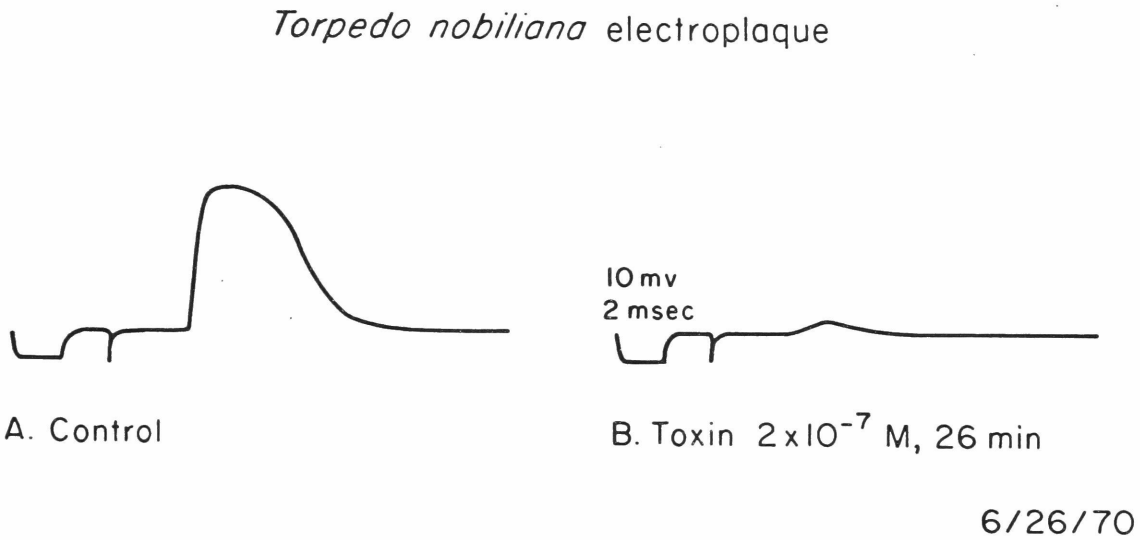


Figure III-7. PSPs in a Torpedo electroplaque, before and after toxin treatment.

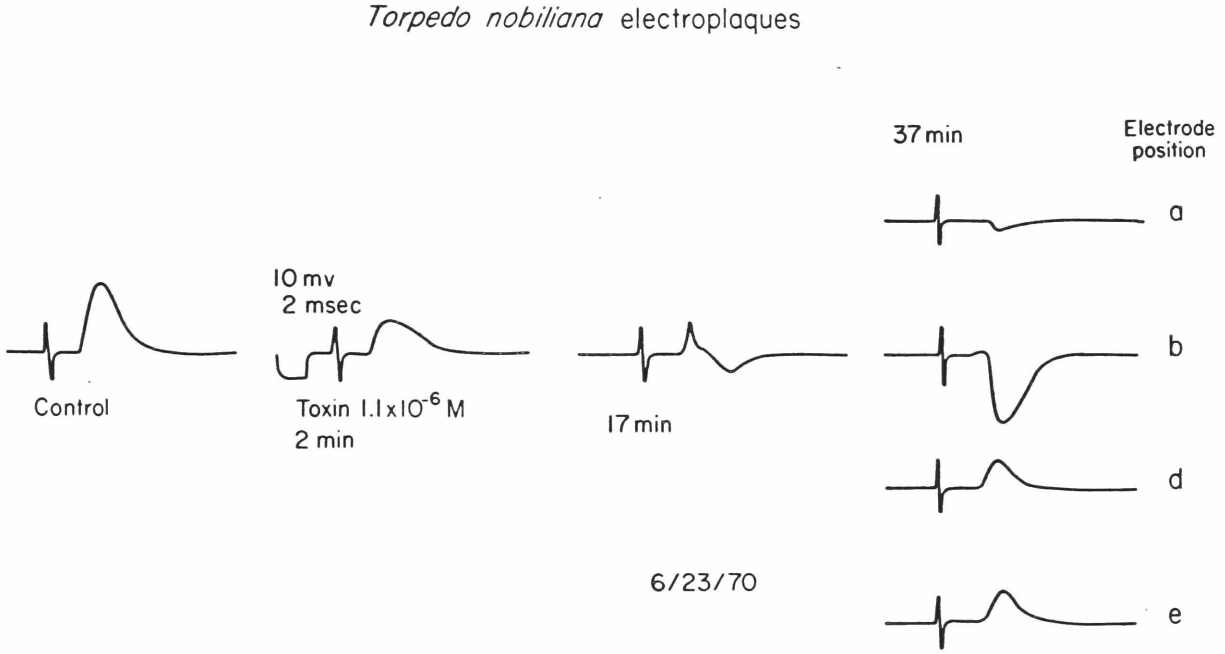


Figure III-8. Tracings of microelectrode recordings from a column of Torpedo electroplaques. Electrode position refers to Figure II-4. Control and responses at 2 and 17 minutes after addition of toxin were made at location b.

and the stronger but later potential differences which arise from postsynaptic currents in subsurface electroplaques (Bennett et al., 1961, Fig. 5H). Similar records were obtained in a study of dTC action in this preparation, suggesting that the smaller dTC molecule also fails to penetrate to deep cells (Bennett et al., 1961).

Aplysia californica Abdominal Ganglion

Figure III-9 shows that relatively high concentrations of the toxin (6×10^{-7} M) applied for long time periods (5 hours in one preparation) had no significant effect on the cholinergic PSP under investigation. In the experiment shown in Figure III-10, subsequent application of dTC caused a reversible diminution of the PSP. This confirms its cholinergic nature.

Isolated Frog Heart

The toxin had no effect on the frog heart. Figure III-10 presents results of a typical experiment. The toxin treatment would have decreased the ACh sensitivity at the frog endplate by several orders of magnitude. Control applications of ACh cause a weak, erratic heartbeat (curve A); toxin alone (B) has no effect; the response to ACh occurs with undiminished intensity after treatment with toxin (C).

Atropine, a muscarinic antagonist, still blocked ACh sensitivity after toxin treatment (Curve D). Epinephrine does not act on cholinergic receptors; as expected, the toxin also had no effect on this drug's excitatory action (Curve E).

Discussion

The experiments on the frog myoneural junction show that cobra toxin acts solely on ACh sensitivity, although in many cases the analysis of quantal content is complicated by the toxin's non-uniform action along an individual endplate. These results agree with the

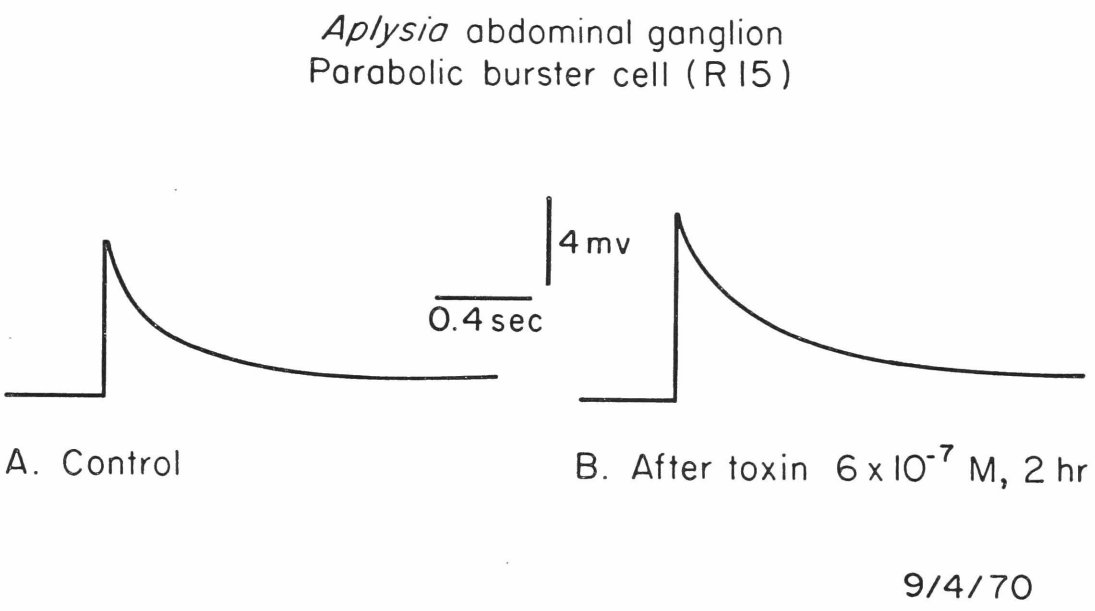


Figure III-9. Tracings of PSPs in Aplysia neurones, as described in the text.

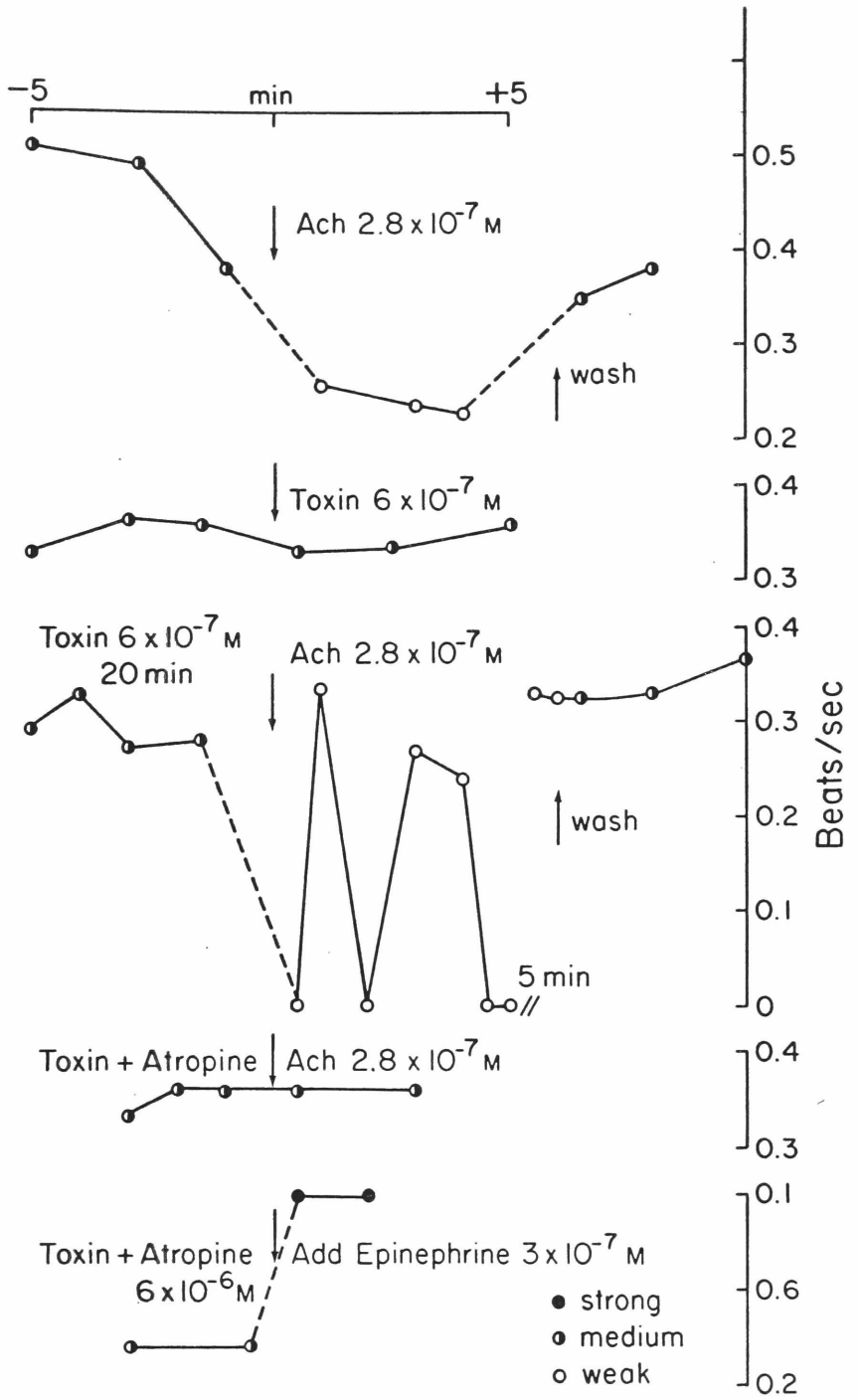


Figure III-10. Response of the isolated frog heart to cobra toxin and reversible drugs, as described in the text. Test substances were added at the vertical arrow; substances written to the left of the arrow were already present in the Ringer.

observations of Meldrum (1965a) and of Chang and Lee (1966), who employed the same preparation, and with studies using less specific techniques on other vertebrate myoneural preparations (reviewed in Chapter I).

Electroplaques show nicotinic pharmacology (see p. 17 and review by Bennett, 1970). My results, that the toxin blocks transmission in Raja and Torpedo electroplaques, provide support for the hypothesis that the toxin acts on all nicotinic receptors.* Also, bungarotoxin blocks transmission at Electrophorus (Changeux et al., 1970) and Torpedo electroplaques (Miledi et al., 1971).

The toxin has no detectable action on the frog heart, and bungarotoxin failed to affect the guinea pig ileum (Chang and Lee, 1963). Thus, muscarinic receptors, whether inhibitory (heart) or excitatory (ileum) show no susceptibility to the toxin. All these facts suggest that the toxin blocks only nicotinic receptors.

This conclusion is strengthened by my observations that the toxin fails to block a cholinergic PSP in a mollusc, Aplysia californica. These ACh receptors can be classified as neither nicotinic nor muscarinic (see p. 5). In this connection, it would be interesting to know the toxin's action on an invertebrate synapse which shows nicotinic pharmacology, such as the leech myoneural junction (Flacke and Yeoh, 1968a, b).

---

*One report (Chou and Lee, 1969) is at variance with this conclusion. Cobra toxin failed to block ganglionic transmission, either in vivo (cat superior cervical ganglion) or in vitro (guinea pig hypogastric nerve-vas deferens preparation), as measured by muscle tension at the nictitating membrane or vas deferens.

Three caveats prevent the simple conclusion that ganglionic receptors are invulnerable to the toxin. First, it was not clearly shown that the toxin reached the vicinity of the postsynaptic receptors. Second, the relation between ganglionic PSP amplitude and muscle tension is unknown; intracellular postsynaptic records would be possible and desirable. Third, autonomic ganglia show, in addition to the nicotinic EPSP, a slow muscarinic component (Eccles and Libet, 1961) whose mechanism is not easily explained by ionic permeation (Libet, 1970). Under certain conditions, this second EPSP reaches spike threshold (Nishi and Koketsu, 1966; Brown, 1967). (continued)

Finally, despite the fact that ganglionic receptors are classified as nicotinic on the basis of their susceptibility to dTC, their pharmacology shows significant differences from that of the myoneural junction. One of these differences involves the members of the bis-onium series (see p. 8); others are reviewed at length in the pharmacological literature (for instance, Goodman and Gilman, 1965). If cobra toxin can in fact distinguish between ganglionic and myoneural ACh receptors, this action would not be unprecedented.

CHAPTER IV

QUANTITATIVE STUDIES ON POTENCY

To explore the mechanism of cobra toxin's action, one requires a reproducible assay for the potency of the toxin. Figure IV-1 shows the basis for such an assay.

The Inactivation Rate Constant α

As previously noted (p. 31), the toxin appears to act with first-order kinetics, irreversibly inactivating a given fraction of the receptors per unit time. In agreement with this mechanism, Figure IV-1 shows an exponential decline in the EPP after the chamber is flushed with Ringer containing toxin. The decline halts, but fails to reverse, when the chamber is flushed with toxin-free solution. When the preparation is again subjected to the same toxin concentration, the decline resumes with the same slope.

For this exponential decline, we may measure a time constant, τ, such that the EPP falls to 1/e of its original amplitude in time τ. The slope of the descending straight line in Figure IV-1 is then proportional to $\alpha = 1/\tau$, and we may define α as the rate constant for the inactivation process.

α as a Function of Toxin Concentration

Figure IV-2 shows α versus toxin concentration for 47 endplates, each represented by a point. The lowest concentration produced results like those shown in Figure III-1; the highest concentration is represented by Figure III-3.

As discussed in Chapter III, there was appreciable scatter in α among endplates tested with the same concentration of toxin. It will be shown in the Discussion (p. 61) that this scatter probably occurs because toxin molecules must traverse varying diffusion paths

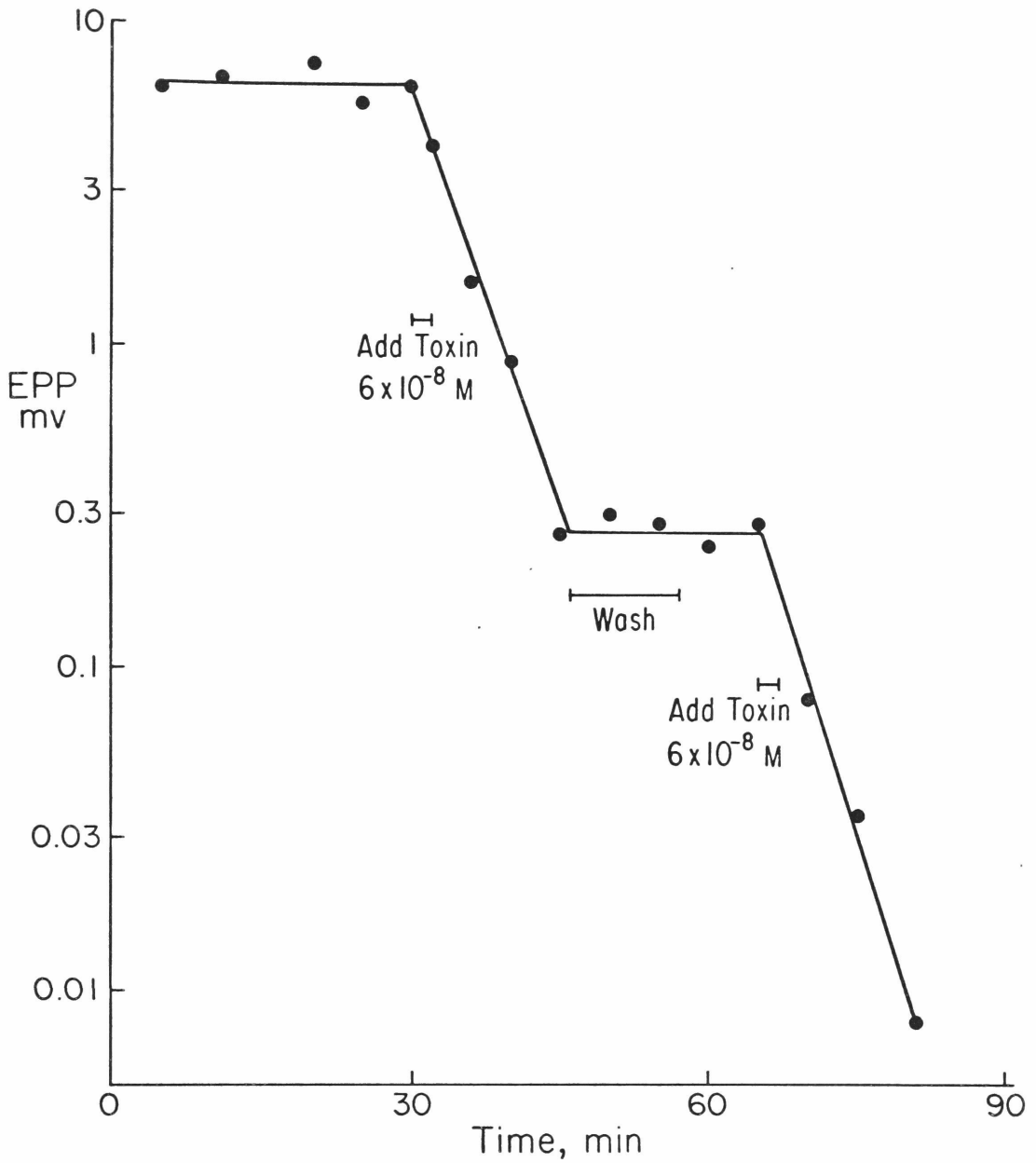


Figure IV-1. Exponential decrease in the EPP as a reproducible assay for the cobra toxin's potency. Each point represents the average of 64 endplate potentials (EPPs), except for the last two which represent 128. Horizontal lines represent times when chamber was flushed with new solutions.

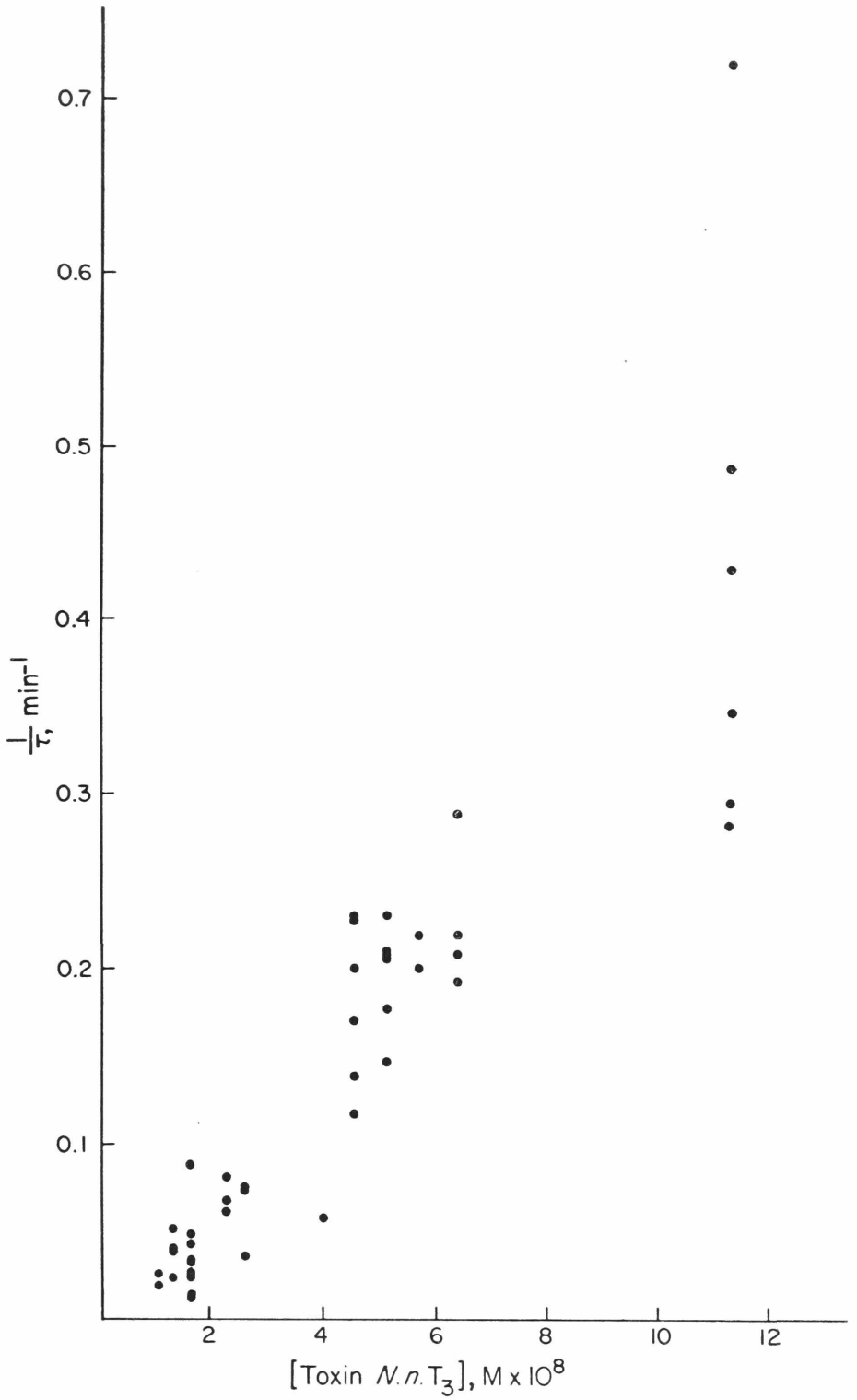


Figure IV-2. Potency vs. toxin concentration for 47 different endplates measured over a one-year period. Experiments like that of Figure IV-1 were used to determine $1/\tau$.

to reach the receptors. Nevertheless, α clearly increases with concentration. The results fit a proportional relationship with a constant k_T of approximately 3.3×10^6 min^{-1} M^{-1}, or 5.5×10^4 sec^{-1} M^{-1} (see Eq. IV-1, p. 58).

Extending the Concentration Range It would be interesting to extend the dose-potency results to higher and lower toxin concentrations than shown in Figure IV-2. At higher concentrations extrapolation predicts that α would increase to greater than 1 min^{-1}. Under such conditions, my apparatus would not allow fluid changes rapid enough to establish a steady concentration before the EPP declined to unmeasurable levels.

For lower concentrations, one extrapolates to $\alpha = 3 \times 10^{-3}$ min^{-1} at toxin concentrations of 10^{-9} M. I have observed that α continues to decrease as the toxin concentration decreases. However, quantitative measurements on α become unreliable in this range, because of several perturbing processes. For instance, the EPP often changed in amplitude during solution changes, sometimes as much as a factor of 2. These changes seem mainly to involve the quantal content and may reflect mechanical stresses on the nerve terminal (see Goldsmith, 1963). Many control preparations also showed additional slow drifts in quantal content. The much smaller decline caused by the toxin could not be measured against these background processes.

Latency as a Function of Toxin Concentration

This point was not studied systematically, because the methods for changing solutions improved gradually over the course of these experiments. These modifications undoubtedly affected latency measurements. Nevertheless, some observations are possible.

Let us define latency as the time from the start of the solution change until the fractional rate of change of EPP amplitude,

$\frac{dEPP}{EPP}$, reaches 90% of its final value. This final value is α, and we measure latency by noting the time required for the semilogarithmic plot of EPP vs time (Figures III-1, III-2, III-3, IV-1) to attain linearity. The Discussion (p. 61) presents a natural basis for this formulation in terms of diffusion processes.

At any given toxin concentration, latency varied by at least a factor of 3, as did α. The two variables were often--but not always--correlated: a short latency accompanied a large α, and vice-versa.

Despite the uncertainties introduced by changing methods and by scatter in the data, it was clear that latency decreased with increasing toxin concentration. In Figures III-1 and III-2, the latencies are about 50 min; at this toxin concentration (1.6×10^{-8} M), the range was 10-60 min. This is the lowest toxin concentration that produced reliable latency measurements. For the highest concentration tested (1.1×10^{-7} M; see Fig. III-3) the range was 160 to 480 sec (average, 320 sec); the solution change required 150 sec for completion in this case.

Temperature Dependence of α at the Skate Electroplaque

The large electroplaque cells in the skate may be penetrated repeatedly and withstand the mechanical stresses that accompany large temperature changes. I therefore used this preparation to study the effects of temperature on the toxin's potency. Figure IV-3 shows α for 15 preparations tested at constant temperatures in the range 7-30°. As with the frog myoneural junction α varied greatly among preparations.

Decrease in α at Low Temperatures The toxin seems unable to inactivate receptors below about 11°. Three factors could conceivably account for this loss of potency. First, some physical change could occur in the toxin molecules below 11°. No such change, however, has

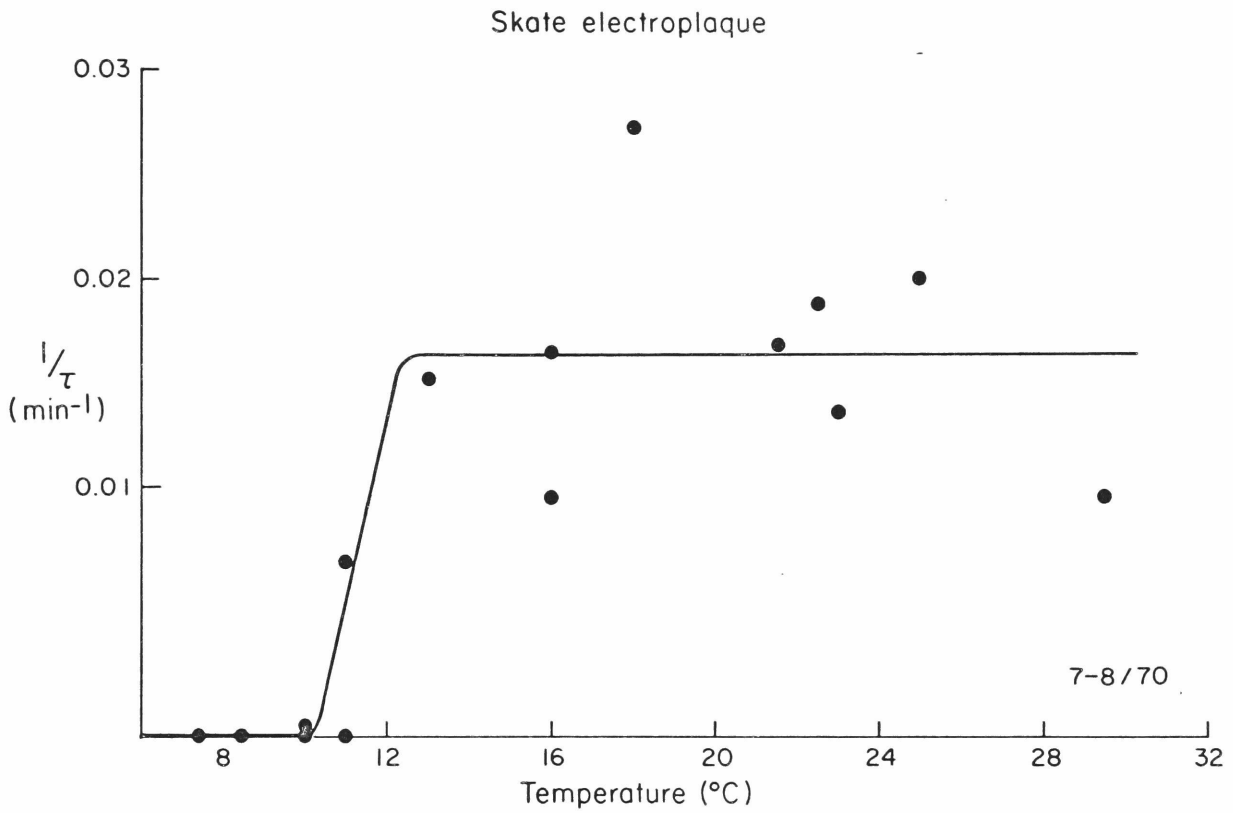


Figure IV-3. Potency of the toxin vs. temperature for the skate electroplaque. Determination of $1/\tau$ followed the same procedures as for the frog myoneural junction.

been observed in physical-chemical studies with the toxin (Chapter I). Second, the receptor molecules might change. Some studies do show temperature-dependent alterations in the macroscopic properties of membranes, presumably resulting from phase changes (Ladbrooke et al., 1968; Reinert and Steim, 1970). But there are no discontinuities in the ACh-receptor interaction at the myoneural junction (Katz and Miledi, 1965d; Steinbach, 1971). Finally, the extracellular tissue near the postsynaptic membrane may change at low temperatures so as to become impermeable to the toxin. It should be mentioned in connection with this idea that dTC showed no large change in its blocking activity in the skate electroplaque below 11°. Assuming a partial specific volume of 0.71 cm^3/g for the toxin (Karlsson et al., 1966), its diameter is 25 Å; the longest dimension of dTC is about 15 Å (Dr. H.M. Sobell, personal communication). The two molecules thus have comparable sizes, but several types of physical barriers would be able to distinguish between them. The origin of the 11° cutoff remains unexplained.

Lack of Temperature Effects Above 11° The results in Figure IV-3 are inconsistent with a Q_{10} greater than about 1.5 in the range 12-30°. Because of the experimental scatter, however, it is impossible to determine a more definite value for the Q_{10}. In Figure IV-3 a line has been drawn horizontally through the average of all points above 12°, in order to emphasize that the toxin's action shows no marked temperature dependence.

Discussion

In this section, we first propose a simple model for the toxin's action, then discuss the evidence for the model. The model is:

$$T + R \xrightarrow{k_T} TR. \qquad \text{IV-A}$$

A toxin molecule T and a receptor R form the irreversible, inactive complex TR. This scheme leads to the differential equation:

$$\dot{r}_1 = -k_T[T]r_1, \qquad \text{IV-1a}$$

where r_1 is the number of available receptors R (the subscript 1 is included for consistency with Chapter V) and [T] is the toxin concentration near the receptors. (It will be shown that [T] is nearly equal to the toxin concentration in the bathing solution.) When [T] is constant, we define

$$\alpha = k_T [T] . \qquad \text{IV-1b}$$

This chapter describes the kinetics of EPP and PSP diminution during toxin action. Two quantitative questions merit consideration. First, what is the relationship between the EPP (or PSP, in the skate) and the number of available ACh receptors? Second, how does the toxin's concentration in the synaptic cleft compare with that in the bathing solution? After discussing these points, we shall be in a position to examine the molecular interpretation of Scheme IV-A.

EPP as a Measure of Receptor Number The relation between dose and response has taken on importance with the recent rise of quantitative pharmacology; Waud's (1968) review summarizes current methods and ideas. "Receptor occupancy" is a fundamental concept. Consider a simple monomolecular binding of ACh to the receptor, R:

$$\text{ACh} + \text{R} \underset{}{\overset{a}{\rightleftharpoons}} \text{Ach}\cdot\text{R}, \qquad \text{IV-B}$$

where Ach·R is the "occupied receptor" or "ACh-receptor complex." If a is the affinity constant for the binding, the law of mass action implies that

$$[\text{ACh}\cdot\text{R}] = \frac{ar_1[\text{ACh}]}{1 + a[\text{ACh}]} . \qquad \text{IV-2}$$

Equation IV-2 has the same form as the Michaelis-Menten expression or the Langmuir adsorption isotherm. Several lines of evidence lead to the conclusion, for normal neuromuscular transmission, that $a[ACh] \ll 1$ (Waser, 1960; Waud, 1967; Steinbach, 1968b). Therefore, with good accuracy,

$$[ACh \cdot R] = ar_1[ACh]. \qquad \text{IV-3}$$

The number of occupied receptors is thus proportional to the ACh concentration and to the number of available receptors.

Next, we consider the relation between receptor occupancy and the observed effect--in this case, the EPP amplitude. Stephenson (1956) and others have pointed out that this relation need not be a linear one. "Spare receptors," for instance, would mean that the response amplitude reaches saturation even though some receptors remain in the R state.

At the frog myoneural junction, however, the only significant nonlinear contribution to the occupancy-EPP relation arises because depolarizations displace the electrochemical driving force for the endplate current. In my experiments, all EPPs of greater than 1 mv peak amplitude were corrected for this process with Martin's (1955) formula:

$$EPP = EPP' \frac{1}{(E_D - EPP')} \qquad \text{IV-4}$$

Here EPP is the corrected amplitude; EPP' represents the observed amplitude. The driving force E_D is the difference between the resting potential and the reversal potential for the endplate current; this reversal potential is about -15 mv (del Castillo and Katz, 1954f; Takeuchi and Takeuchi, 1959; 1960).

Katz and Thesleff (1957) observed another source of non-linearity in the occupancy-EPP relation. They measured the depolarizations produced by ACh or Carb pulses from each barrel of twin

micro-ionophoretic pipettes. They then applied the two pulses simultaneously and obtained an depolarization whose amplitude exceeded the sum of those produced by the two pulses individually. This result expresses an occupancy-EPP curve which is concave upwards, or "S-shaped," rather than linear. The nonlinearity could arise from several sources. First, the affinity constant, a, might vary over the receptor population. Second, some binding site other than the receptor could take up limited quantities of agonist (Katz and Thesleff, 1957) (this mechanism receives more attention below, p. 63). Finally, the cause might also be certain idiosyncracies in the method of micro-ionophoretic application (Waud, 1967). The last explanation seems likely because the nonlinearity fails to appear when receptor occupancy is defined by the amount of charge transferred through the pipette (Waud, 1967) rather than by the superposition procedure (Katz and Thesleff, 1957). In any case, the effect is small and, following the example of its discoverers, we shall ignore it for quantitative analyses of receptor characteristics (Katz and Thesleff, 1957).

The EPP is, of course, not a steady potential but has a complex waveform. Its amplitude and time course are determined by the endplate current (Takeuchi and Takeuchi, 1959; 1960) and by the cable properties of the muscle fiber (reviewed by Falk and Fatt, 1965). The endplate current in turn depends upon the detailed time course of ACh acetylcholine's release from the presynaptic terminal (Katz and Miledi, 1965b, d), its diffusion, its hydrolysis by acetylcholinesterase (del Castillo and Katz, 1955, 1957b), and the stability of the ACh·R complex. We merely emphasize here that all these processes are also linear with the amount of ACh, for my experimental conditions.

Our conclusion is that, upon correction with Eq. IV-4, the following relationship holds:

$$EPP = \nu[A]r_1 \qquad \text{IV-5}$$

where ν is a constant. $[A]$ represents the total amount of agonist

(ACh or Carb), released either from the nerve terminal or from a micro-ionophoretic pipette, when the time course of release remains constant. Equation IV-5 therefore expresses the important fact that the EPP (or drug depolarization) varies linearly both with the amount of agonist and with the number of available receptors.

Toxin Concentrations Near the Receptors As pointed out briefly in Chapter III (p. 34), the synaptic cleft is a complex, torturous structure of largely unknown physical characteristics. Diffusion processes, however, play a venerable role in biophysics. Impressive intellects have studied the topic and their conclusions can generate some insights on the toxin-receptor interaction.

That the toxin concentration near the ACh receptors does not instantaneously follow the bath concentration may be deduced from two observations. First, a finite latency ensues between the introduction of toxin to the bath and the first effects on the EPP in surface fibers (Figs. III-1, II-2, II-3). Second, many interior fibers show little diminution in their EPP even after EPPs in surface fibers have been reduced by a factor of 100.

This last fact suggests a means to attack the problem. Assume that the central plane of the muscle is a perfect sink for toxin molecules--at least for times on the order of hours.* Let the muscle be an infinite slab exposed to the solution at both surfaces; the upper surface (where we impale fibers) lies at $x = d$, the lower surface at $x = -d$. We assume for the moment that toxin molecules undergo only diffusion within the muscle. The diffusion equation for the steady state is given by

---

*Hill and Macpherson (1954) considered diffusion in the frog sartorius muscle without this assumption. Their table I allows the reader to draw conclusions very similar to the one presented here. For our parameters, $\frac{K\pi^2}{b^2}$ equals 1.5×10^{-4} sec^{-1}.

$$\frac{d^2[T]}{dx^2} = 0, \qquad \text{IV-6}$$

and for $[T] = [T_o]$ in the bathing solution, we have

$$[T] = [T_o]\frac{|x|}{d} \qquad \text{IV-7}$$

at any distance x from the central plane. Equation IV-7 holds for the steady state.

The approach to the steady state is described quite usefully by Jacobs (1967, pp. 62-63). The time t required to attain 90% of the final toxin concentration is given by

$$t = \frac{0.3(d-x)^2}{D_m} \qquad \text{IV-8}$$

and is independent of T_o. Here D_m is the diffusion coefficient inside the muscle (see below). We now take reasonable values for the parameters in Equations IV-7 and IV-8 and ask whether these equations provide a good description of the kinetics of toxin action. Assuming that Equation IV-1 holds, the fractional rate of change of EPP amplitude, $\frac{\frac{dEPP}{EPP}}{d_t}$, is proportional to [T]. This rate of change is the slope of a semilogarithmic plot of EPP vs. time (Figs. III-1, III-2, III-3, IV-1); the linearity of these plots constitutes the major evidence for Scheme IV-A.

For the frog sartorius muscle, 2d = 0.07 cm. For molecules ranging in size from inorganic ions (Hill and Macpherson, 1955) to albumin (MW = 7.2×10^4) (Hill, 1964), D_m in the extracellular muscle space is about one-fourth its value in free solution. The diminution arises primarily from the fact that molecules must travel along complex paths. The diffusion constant of toxin T_3 is 1.3×10^{-6} cm^2/sec (Karlsson et al., 1971). Thus, $D_m = 3.2 \times 10^{-7}$ cm^2/sec. For surface fibers of diameter 0.01 cm, x lies between 0.025 and 0.035 cm.

Then, by Equation IV-7, the steady-state value for [T] lies between 0.3 $[T_o]$ and $[T_o]$. Therefore, α (Eq. IV-1b) should vary by about a factor of 3, for any $[T_o]$; this variation was in fact found experimentally. Furthermore, the largest values of α should correspond to a local toxin concentration of $[T_o]$. This point will be discussed in connection with ionophoretic ACh application (p. 64).

The approach to equilibrium, as defined by Equation IV-8, should require from zero to 200 seconds. For the highest toxin concentration tested, the observed values (see p. 55) are only slightly longer than predicted by Equation IV-8 and we conclude that, at this concentration, no other barriers seriously limit the toxin's action.

However, latency increased with decreases in $[T_o]$, reaching a value of 10-60 minutes at $[T_o] = 1.6 \times 10^{-8}$ M (Figs. III-1, III-2). This result violates the simple diffusion scheme we have presented. Perhaps the most straightforward modification is to include a population of binding sites for the toxin. A likely location for the sites would be the extracellular gel that permeates the synaptic cleft and the channels between Schwann, nerve, and muscle cells (see discussion in Chapter III). The binding must be reversible, because bungarotoxin bound irreversibly to only one molecular species--presumably the receptor--in the studies of Miledi *et al.*,(1971).

If the binding is rapid, then the mathematical analysis of diffusion becomes equivalent to that of oxygen diffusing into a muscle with an "oxygen debt." Hill's (1928, Eq. 55) analysis may be adapted to give the following conclusion. Let the equilibration time t be defined for a diffusion barrier as in Equation IV-8. Then, if the barrier also contains binding sites y, the equilibration time t' becomes

$$t' = t \frac{[y]}{[T_o]} . \qquad \text{IV-9}$$

Equation IV-4 expresses the relation, found experimentally, that equilibration time increases as $[T_o]$ decreases. Furthermore, [y] need only be of the order of 10^{-7} M to substantially alter the equilibration time.

These conclusions about α and latency are based upon diffusion processes. They might be tested further with micro-ionophoretic application of ACh. Receptors thus activated are accessible to the external solution across a negligible diffusion barrier (del Castillo and Katz, 1955; Goldsmith, 1963; Katz and Miledi, 1965d). Therefore, α for the toxin's action on ACh potentials should show little variation at a given $[T_o]$ and should not greatly exceed the largest α for EPPs. Furthermore, the latency should equal the time required for solution change. Preliminary experiments verify these predictions.

Our discussion of diffusion processes is now complete, and we conclude that after the initial transient, the toxin concentration [T] near the receptors was nearly equal to the bath concentration $[T_o]$. Dropping the subscript to express the bath concentration, we have the result that the inactivation rate constant α (Eq. IV-1) is equal to $k_T[T]$. From Figure IV-2,

$$k_T = 5.5 \times 10^4 \text{ sec}^{-1} \text{ M}^{-1}.$$

Validity of these Conclusions for the Skate Electroplaque

In the skate, as in the frog, it seems that the toxin concentration near the receptors was nearly equal to that in the external fluid. In comparison with frog muscle, we know relatively little about the diffusion properties of the organ and the biophysics of individual electroplaques. Nevertheless, the important observation was that the PSP declined exponentially after an initial delay comparable to that described for frog muscle.

It is worthwhile to note that the inactivation rate constant α for the skate preparation was some 15 times smaller than for the frog at the same [T] (compare Figs. IV-2 and IV-3). This fact could imply that the toxin had to suffer hindered access to the receptors. However, dTC also has about ten times less potency in this preparation than in the frog (preliminary observations). Therefore, the difference in k_T between the skate and the frog probably arises from some molecular variation in the receptors themselves.

The possibility still remains, however, that the toxin loses it effectiveness below 11^o because a change occurs in the diffusion properties of the extracellular gel, thereby preventing the toxin from reaching the receptors (see Results, p. 56, for a full discussion).

The Toxin-Receptor Interaction Our value of k_T may be compared with the maximum possible rate constant for a reaction between a drug molecule and a receptor. This corresponds to the encounter-controlled reaction, in which each collision is successful and molecules react as rapidly as they can diffuse toward each other. Burgen's treatment (1966) has particular relevance to the drug-receptor interaction. Suppose the receptor engulfs the toxin so that the radius of the complex is not substantially larger than that of the toxin. Then, at 20^o, the encounter-controlled rate constant is 10^9 sec^{-1} M^{-1}. This value exceeds 10^4 times the observed k_T. We should therefore consider processes which may modify the rate constant k. Electrostatic attraction can increase k by at most a factor of 2. However, electrostatic repulsion, the presence of bound water or ions, and the requirement of activation energy for combination could each reduce k by several orders of magnitude. Since the toxin is a large molecule, we also expect that it will probably not be able to display all aspects to the receptor at each encounter; hence, the probability of reaction will be reduced.

Does the Toxin Bind Reversibly? Suppose the toxin-receptor binding is reversible:

$$T + R \underset{k_{-T}}{\overset{k_T}{\rightleftharpoons}} T \cdot R \qquad \text{IV-C}$$

Then k_{-T} is the rate constant for recovery of the EPP after the toxin is washed away. My experiments would have detected a recovery with a time constant of one day. Therefore, $k_{-T} < 10^{-5}$ sec^{-1}.

The dissociation constant for the toxin-receptor complex is given by k_{-T}/k_T, so the dissociation constant must be less than 0.2 nM. The dissociation constant for tetrodotoxin's binding to sodium channels is about 1 nM (Hille, 1968). Therefore, cobra toxin binds to the receptor at least as tightly as this; of course, the actual rate constants are several orders of magnitude smaller in the case of cobra toxin, so its action appears irreversible.

Mechanisms of Toxin-Receptor Binding. In considering Scheme IV-A, it is worth noting that the toxin need not bind directly to R so long as the end result is an inactive receptor. Each toxin molecule might also bind to two or more receptor sites. This possibility draws support from the fact that the toxin's calculated diameter is 25 Å; the distance between adjacent receptors is less than 15 Å if both amine groups in dTC and decamethonium bind to receptors (see Chapter I. Structure-Function Relations).

Could the toxin act like an enzyme? It would chemically inactivate receptors, then diffuse away unchanged. Enzymatic reactions, however, generally have a Q_{10} of greater than 2. The toxin's action, like that of dTC (van Maanen, 1950; Jenkinson, 1960), has a small temperature dependence (above 11°). This fact argues for a mechanism based upon irreversible binding rather than enzymatic catalysis. Also, treatment of rat diaphragm with ^{131}I-bungarotoxin (Lee and Tseng, 1966) or cobra toxin (Tseng et al., 1968) causes

specific retention of the radioactivity at the endplates.

Furthermore, ^{131}I-bungarotoxin has recently been used as a specifically bound chemical marker for the isolation of the ACh receptor from Torpedo electroplaques (Miledi et al., 1971). Since the toxin is quite resistant to proteolysis in its native form (see Chapter I), one doubts that only a peptide fragment remains bound to the receptor structure.

In summary, the most likely mechanism for the toxin's action is that it inactivates receptors by binding irreversibly to the postsynaptic membrane. Chapter V describes experiments which constitute a direct test of this postulated mechanism.

CHAPTER V

COBRA TOXIN BINDS TO ACETYLCHOLINE RECEPTORS

Rationale

Cobra toxin acts specifically with low reversibility to block nicotinic ACh receptors. At the concentrations where this action has received most study (10^{-8} M to 1.2×10^{-7} M), no other physiological effects have been detected (Chapter III).

Furthermore, this toxin, and its homologous peptides in the venoms of other elapid and hydropheid snakes, manifests no enzymatic activity (see Chapter I). Derivatives of these polypeptides, labeled with ^{131}I, have been shown to bind to the endplate regions of skeletal muscle fibers (see Chapter I).

These facts suggest that cobra toxin acts by binding specifically and irreversibly to nicotinic acetylcholine receptor sites on postsynaptic membranes. If such binding occurs, then one should be able to protect the receptors with other compounds--both antagonists and agonists (see p. 6)--which bind to ACh receptors. Furchgott (1964) has reviewed this technique and its applications.

Such an agent is d-tubocurarine (dTC) (Jenkinson, 1960), an antagonist. Using recordings of tension in the frog rectus abdominus muscle, Chang (1960) found that dimethyl-dTC provided the expected protection against crude Bungarus multicinctus venom. Similar observations were soon made with Formosan cobra (Naja naja atra) venom (Su, 1960). Later, it was briefly reported that dTC protected the chick biventer cervicis muscle against the pure toxins from these snakes (Lee and Chang, 1966; Su et al., 1967). Although protection appeared nearly complete in these experiments, recordings of muscle tension provide only an indirect measure of receptor activity. I have extended these results using microelectrode techniques, and I find that dTC protects against the toxin only at high concentrations.

Acetylcholine itself must also bind to the ACh receptor. Several studies have shown that carbamylcholine (Carb) acts precisely like ACh, except that Carb is not hydrolyzed by acetylcholinesterase (del Castillo and Katz, 1957b; Jenkinson, 1960). I report here that these agonists protect against cobra toxin much more effectively than does dTC. This qualitative difference probably arises from the desensitization produced by all agonists.

The effectiveness of a protective agent should depend, to a first approximation, on how well it binds to the ACh receptor. Like all potent agonists and antagonists, choline contains a quaternary ammonium group. I have therefore used choline as a control protective agent. It seems effective only at concentrations several orders of magnitude higher than those of dTC, ACh, or Carb. This finding is consistent with the known weak effects of choline on ACh receptors (del Castillo and Katz, 1957c).

Desensitization

Several investigators have described the postsynaptic response to prolonged application of agonists either by perfusion (Fatt, 1950; Thesleff, 1955) or ionophoretically (Katz and Thesleff, 1957). Figure V-1 illustrates the response, known as "desensitization." When Carb is added to the bath, the membrane potential rapidly approaches the reversal potential for ACh action. During this time, muscle fibers twitch; hence, microelectrode penetrations are impossible to maintain. After a few minutes, the membrane potential begins returning to normal resting level. In this phase, the ACh receptors no longer produce an increased ionic permeability in response to ACh (Manthey, 1966). Consistent with this observation is the fact that presynaptic nerve impulses no longer elicit detectable EPPs (smaller doses of Carb do allow small EPPs to be observed; see Figures V-6, V-7). The receptors are said to be desensitized. A small steady-state depolarization (10-15 mv) remains

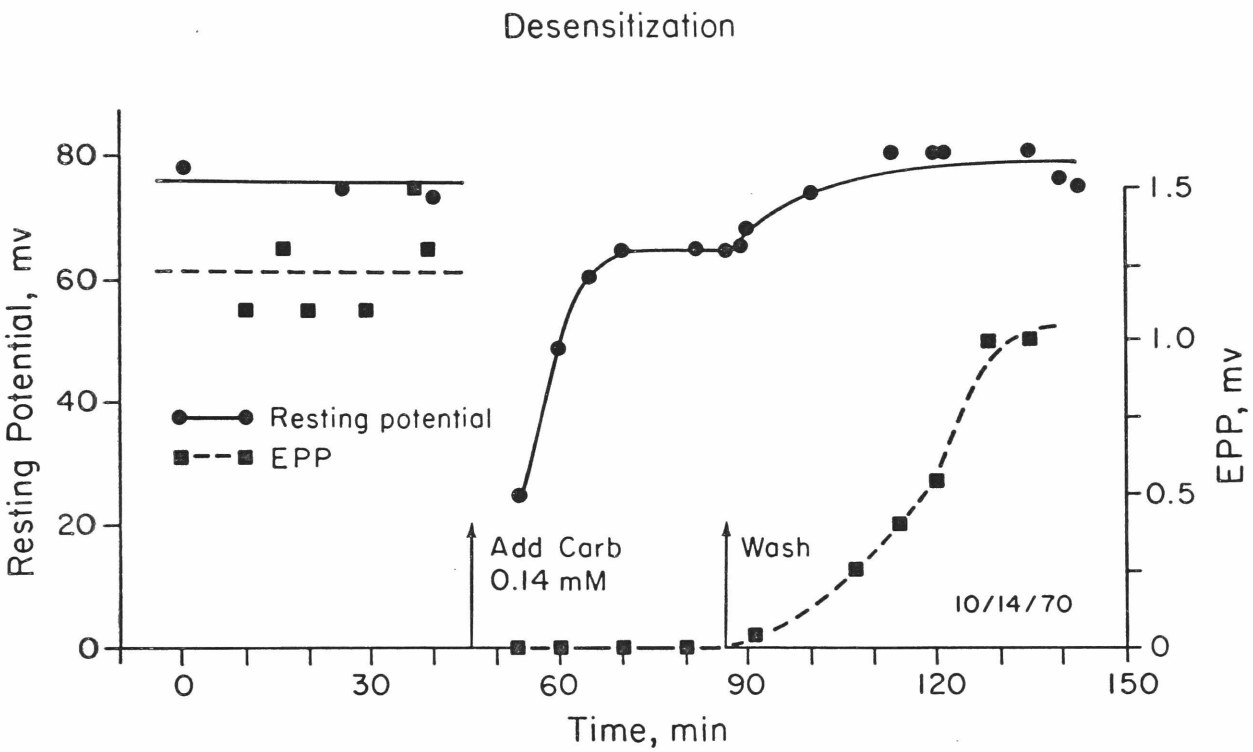


Figure V-1. Desensitization of an endplate by Carb, and recovery. Intracellular recordings were impossible for the first 8 minutes after Carb was added to the chamber, because many fibers were twitching.

in the desensitized state. This steady depolarization is probably not due to endplate receptors (Nastuk and Gissen, 1965; Portela et al., 1970), but occurs all along the muscle fiber. It disappears upon washing with toxin-free Ringer. The EPP also recovers with washing.

The time course of desensitization and recovery depend upon the Carb or ACh concentrations (Fatt, 1950; Katz and Thesleff, 1957; Nastuk and Gissen, 1965; Nastuk and Parsons, 1970; Lambert and Parsons, 1970). The Carb concentration (0.14 mM) used for the experiment in Figure V-1 is approximately that which existed near the receptors in the experiments with ionophoretic ACh and Carb application (see below, pp. 71-75). This concentration was also employed for some experiments with bath-applied Carb as a protective agent (see below, pp. 75-76).

Results of Experiments with Ionophoretic Pipettes

As described previously (see Chapter III), ionophoretic ACh and Carb potentials decrease, as does the EPP, when the preparation is exposed to cobra toxin. Little recovery occurs upon washing with toxin-free Ringer.

Figure V-2 depicts the results of a more complex experiment utilizing an ACh pipette. Trace A shows a control trial with a braking current (del Castillo and Katz, 1955; see also Chapter III). Trace B shows that the receptors near the ACh pipette desensitize (see p. 70), because the ACh potential vanishes. Most of the other receptors, however, are still active, because the EPP has not declined substantially. During ACh application, the fiber depolarizes by a few mV (not shown in Figure V-2). The ACh potential recovers with the re-establishment of a braking current (2-C), and the steady-state depolarization also disappears.

The desensitizing current is then re-applied, and several minutes later the toxin is added to the chamber for 20 minutes. The

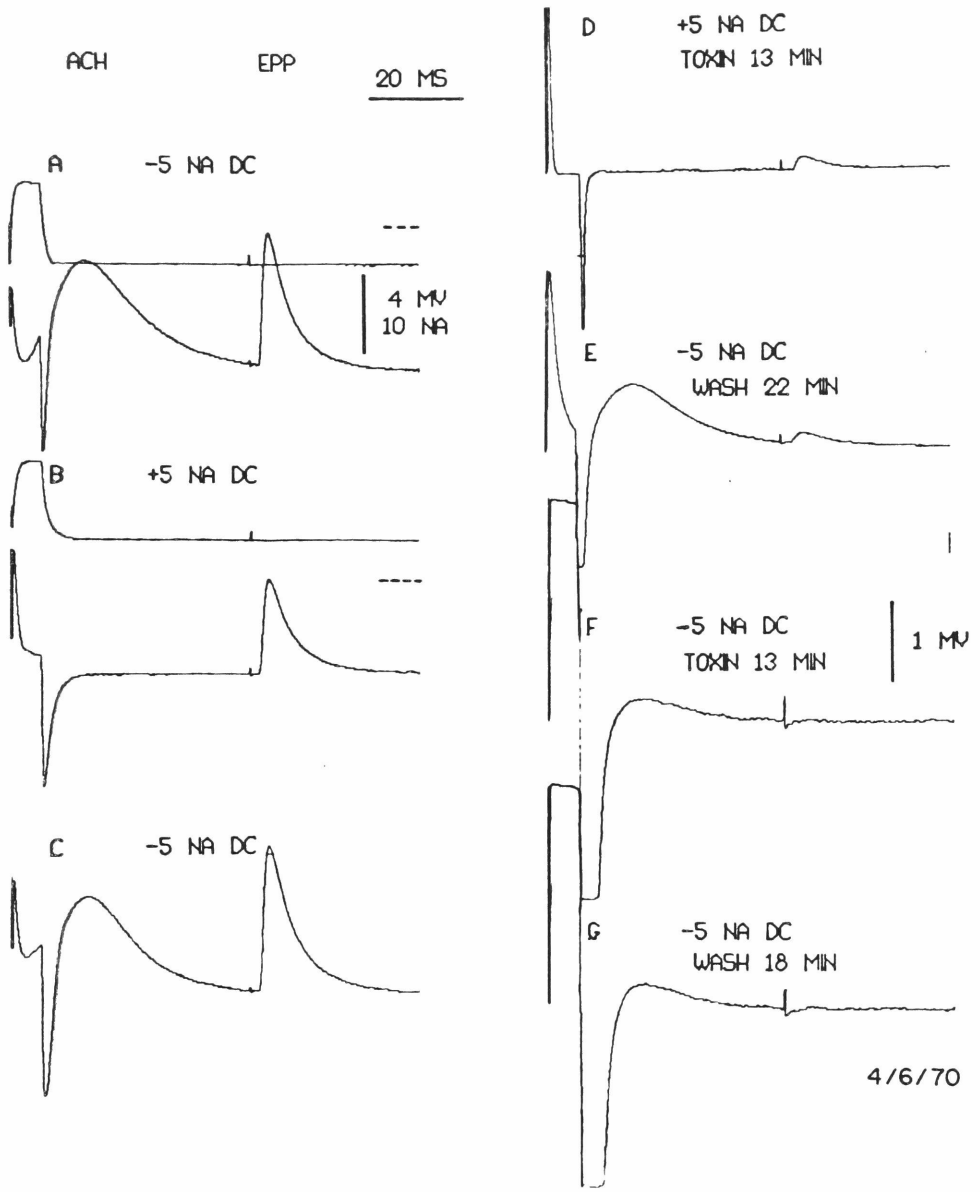


Figure V-2. Focal protection against the toxin by ACh. Procedures as described in text. Each trace represents 16 averaged records. Upper traces, current through ACh-pipette (dashed line denotes zero current); lower traces, intracellular potential. Current trace has been omitted in C-G because it is identical to A or B. Resistance of ACh pipette increased with time, so electrical pickup in recording electrode increased. Note scale change at F.

EPP declines gradually during this period (Figure 2-D). The chamber is then flushed with toxin-free solution, and several minutes later the braking current is re-established. The receptors near the ACh pipette recover from desensitization, as trace C shows.

Furthermore, the ACh potential has been substantially unaffected by the toxin (Trace E). The EPP recovered only slightly in this experiment. In other experiments, however, the recovery was more substantial (Table I), and the reasons for this variation are described below (pp. 74-75).

An important control then consists of adding the toxin in the presence of a braking current. This repeats the experiment described in Figure III-4. As found previously, both the ACh potential and the EPP decline irreversibly (Fig. V-2F, G).

Table II presents the results from four such experiments. It is clear (1) that desensitization itself caused no consistent changes, and (2) that desensitization protected against the toxin at least for the receptors near the drug pipette.

Extent of Protection The extent to which desensitization protected the whole endplate is shown by the behavior of the EPP. This protection varied markedly. On the average, the drug potential was protected more than the EPP, but in some cases the whole endplate seemed protected.

These results are consistent with several idiosyncracies of this technique. Most importantly, endplate geometries vary as to nerve branches and folds (Kühne, 1887; Couteaux, 1947; Cole, 1955). It is thus necessary to deduce what fraction of each endplate was actually desensitized (and therefore protected) by the ACh and Carb applications. Such data are in theory available from the records of resting potential and EPP during desensitization. However, slow drifts in voltage caused by electrode polarization, and unknown behavior of the membrane resistance, actually make this an unreliable

Table II

Protection against cobra toxin by focal ionophoretic application of ACh and Carb. Traces from experiment identified 4/06/70 are shown in Figure V-2. Experimental procedure is described in text. Toxin concentration was 5×10^{-8} M (4/06/70) or 6.2×10^{-8} M (others).

Identifier	Protecting drug	I. Desensitization						II. Protection				III. No Protection			
		Drug potential, mv			EPP, mv			Drug potential, mv		EPP, mv		Drug potential, mv		EPP, mv	
		Before treatment	After treatment	Ratio, after/before	Before	After	Ratio, after/before	After	Ratio after/before	After	Ratio after/before	After	Ratio after/before	After	Ratio after/before
4/06/70	ACh	6.5	4.8	0.74	6.5	7.1	1.09	3.5	0.73	0.6	0.085	0.3	0.09	<0.05	<0.1
4/13/70	Carb	2.5	3.5	1.4	0.9	0.6	0.67	1.2	0.34	0.25	0.42	0.07	0.06	0.03	0.1
4/17/70	Carb	5.5	5.5	1.0	1.0	1.0	1.0	13.5	2.4	0.4	0.4	1.4	0.10	0.04	0.1
4/24/70	ACh	5.5	Not desensitized		6.6	-	-	2.4	0.44	1.5	0.22	<0.05	<0.05	0.05	0.03

practice. Some variation may also have arisen from mechanical drifts in the ionophoretic electrodes, even though care was taken to avoid these effects.

These problems manifested themselves even more clearly during attempts to protect the receptors utilizing choline or dTC pipettes. These experiments required two ionophoretic pipettes, and it proved impossible to obtain long-term stability.

It is therefore impossible to determine the geometrical extent of protection in the experiments described in Table I. To obtain one rough estimate, one can calculate from the rising phase of the drug potentials, that the ACh and Carb pipettes were located about 10 μm from the nearest receptors (del Castillo and Katz, 1955). In the steady state, Fick's law states that ACh concentration decreases inversely with distance from pipette tip. Assuming a transport number of 0.3 and a diffusion constant of 8×10^{-6} cm^2/sec for ACh (del Castillo and Katz, 1955), the ACh concentration at the receptors was 0.75 mM. Other experiments (see below, p. 79) show appreciable protection with concentrations as low as 7 μM, or 1/100 of the concentration at the receptors. Partial protection could therefore have occurred at distances up to 100 times 10 μM, or 1 mm, from the pipette. The longest endplates attain about this length. It is, therefore, not surprising that Table I indicates that the entire EPP sometimes received protection from the Carb or ACh pipettes.

Results with Bath-Applied Protective Drugs

Some uncertainties in the ionophoretic method were overcome by using bath-applied Carb, dTC, and choline as protective agents.

Protection with Carbamylcholine The first series of experiments confirmed the protection afforded by Carb. Because of the large scatter among the characteristics of different endplates, unambiguous protection was obtained only with fairly high doses of both toxin and protecting agents. One major drawback was that the

fibers required several hours' washing for a complete recovery from the reversible blocking agents.

Figure V-3 diagrams a protection experiment, utilizing the same Carb concentration (0.14 mM) as Figure V-1 and a toxin concentration of 1.1×10^{-7} M. Note that a control preparation recovers completely from the Carb treatment alone. Another preparation shows an irreversible blockade of 99% of the receptors after toxin alone (this potency is consistent with the dose-response relationships shown in Figure IV-2). When the Carb and toxin are applied together, however, the EPP declines by a much smaller percentage than for toxin alone. Subsequent applications of toxin alone decreased the EPP by a factor equal to that of preparations in which the first exposure was to toxin alone. This observation was repeated on three different muscles and it confirms the conclusion, drawn from the ionophoretic experiments, that Carb protects against the toxin. Finally, the last experiment shows that Carb treatments cannot reverse a block caused by toxin.

Protection with d-Tubocurarine Figure V-4 shows a strictly analogous experiment utilizing dTC at a concentration (26 μM) which reversibly reduced the EPP to 0.4% of its control level. Toxin concentration was 1.1×10^{-7}M, as in the experiments with Carb. As with Carb, the preparation recovers completely from dTC alone. Furthermore, dTC seems to protect fibers against simultaneous application of the toxin. Subsequent application of the toxin to the same fibers, without protection, causes a decline similar to that observed for the toxin alone. Like Carb, dTC applied after the toxin does not reverse its effects.

Differences between Carbamylcholine and d-Tubocurarine Treatment At first glance, Figures V-3 and V-4 seem to indicate that dTC and Carb, in the concentrations used here, protected the receptors equally against the toxin. Further analysis, however, unearthed a more complex picture. From the 15 fibers represented by the dTC and Carb experiments, I have selected those fibers in which the recording

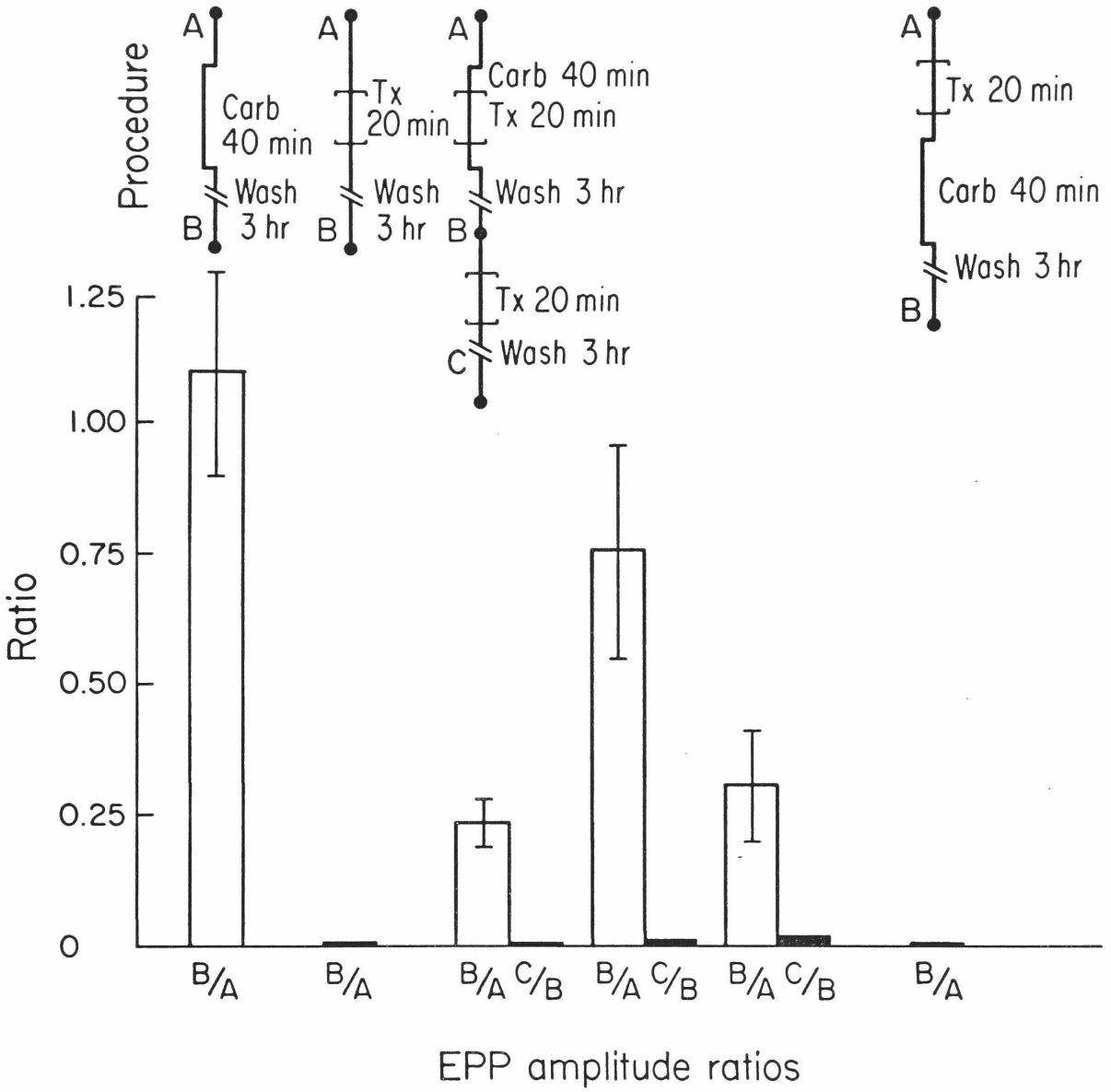


Figure V-3. Protection against cobra toxin by bath-applied Carb. Each ratio (or set of 2 ratios), ± s.d. represents EPP measurements on a different muscle. Five identified endplates were sampled at A, B, and C, as described in the text.

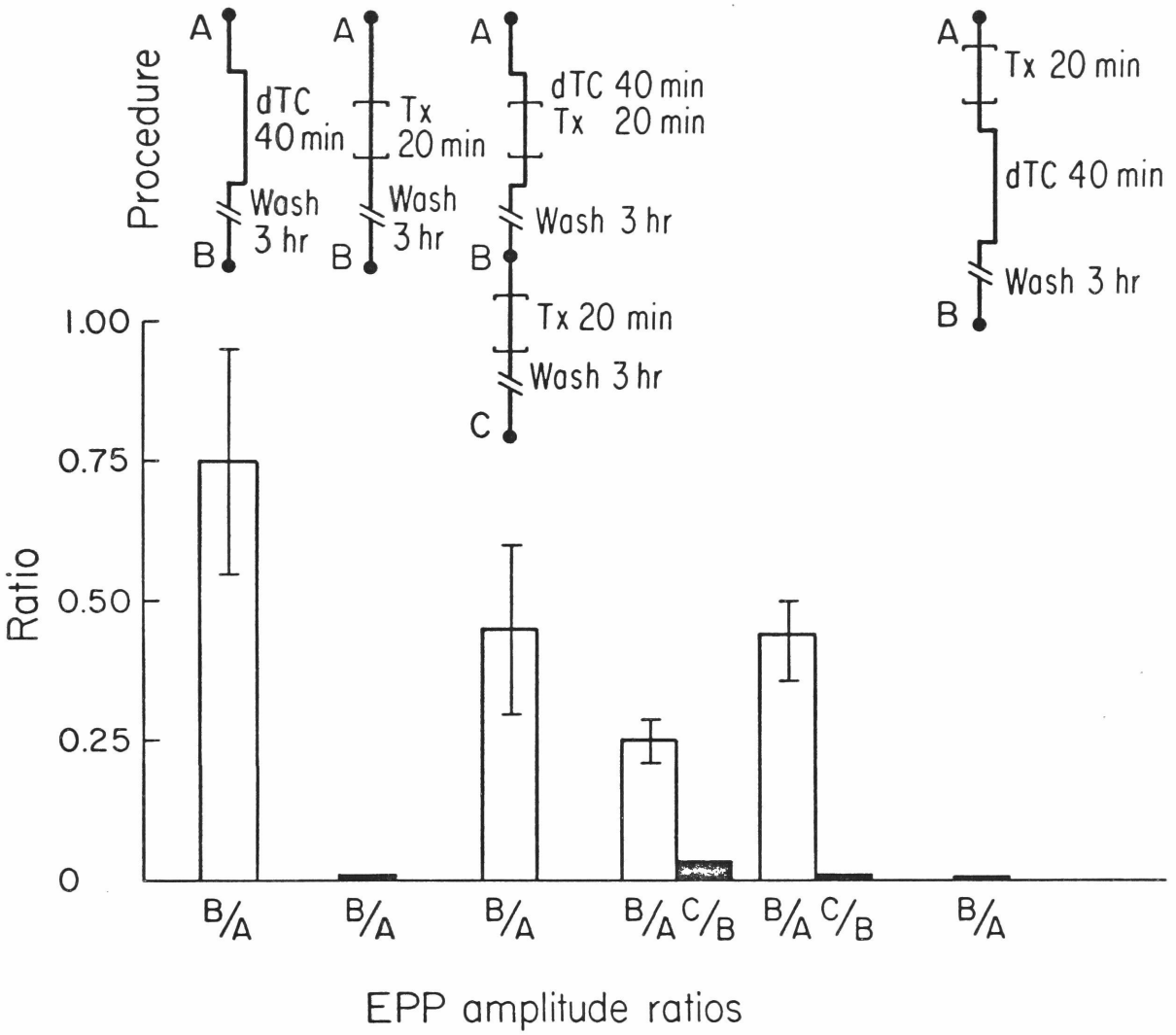


Figure V-4. Protection against cobra toxin by dTC. See text and Figure V-3 for details.

noise, the proximity of the electrode to the endplate, and the MEPP frequency allowed accurate measurements of MEPP amplitude. These amplitudes are plotted in Figure V-5 along with EPP amplitudes in the same fibers. For the Carb protection, the EPP declined by a significantly greater amount ($p < 0.02$, by a T-test) than did the MEPPs. For dTC-protected fibers, however, the two ratios are not significantly different.

Two factors may account for the discrepancy in the Carb case. First, it would seem, from the recovery of the MEPPs (Fig. V-5), that the receptors were completely protected by Carb; all the decline would then arise from a change in quantal content (m). Such a change would not be surprising (Ciani and Edwards, 1963), especially considering the long time courses over which the preparation ran. But such changes did not occur in control preparations treated with Carb alone (Figure V-3) or with toxin alone (Lester, 1970b; Chapter III). A second explanation may therefore be considered based upon the fact that the toxin seems to act asynchronously over an endplate (Chapter III). The Carb may have protected part of the endplate completely and part hardly at all. There would thus be a remaining population of large MEPPs and an apparent decline in m. The data at hand seem inadequate to decide between these two possibilities.

Quantitative Differences in Protection

I next performed two different experiments to explore the details of the protecting mechanisms. In the first experiment (Fig. V-6), doses of Carb, choline, and dTC were selected to reduce EPPs reversibly to 0.3-0.4 times the original EPP. Toxin was applied, as in the experiments shown in Figures V-3 and V-4.

Carb provided significantly greater protection than did dTC, and choline fell between the two. These data are consistent with less complete results from preliminary experiments. One must keep in mind uncertainties which attend variations among different muscles.

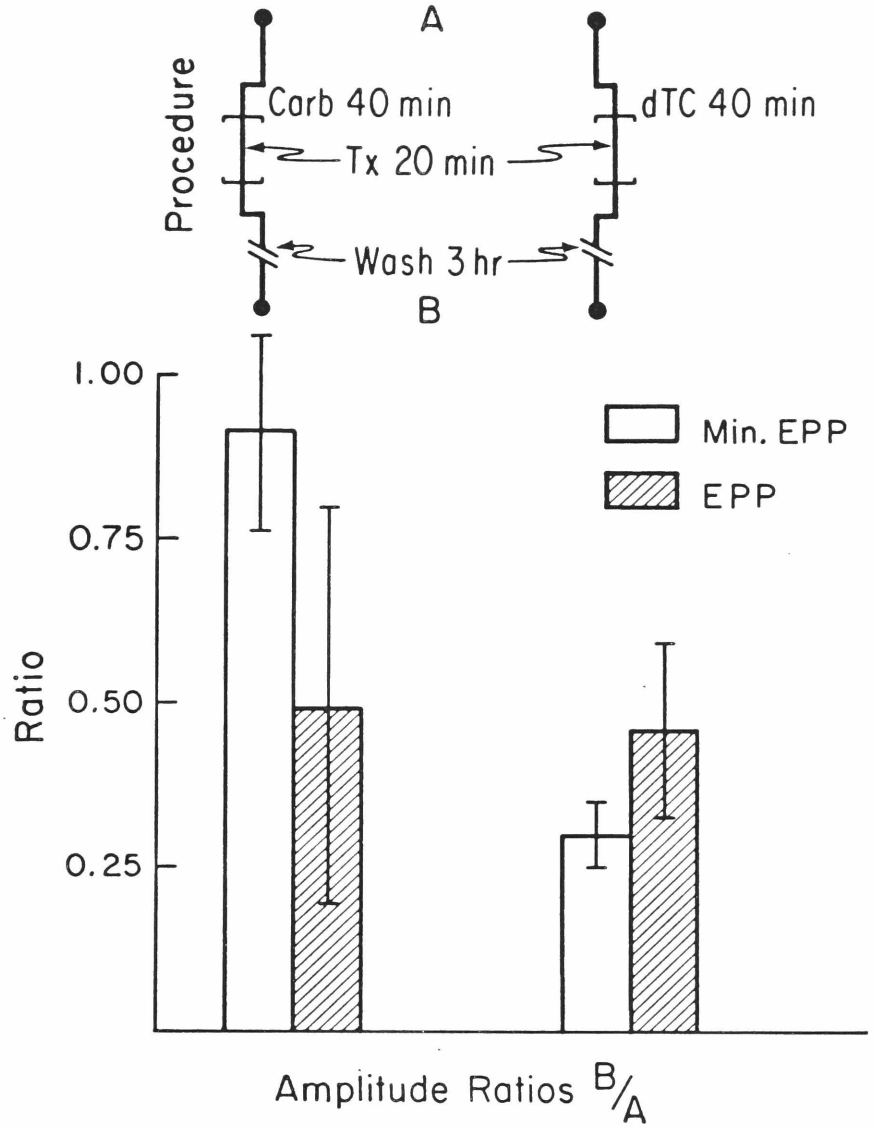


Figure V-5. MEPP amplitudes during the protection experiments (Figs. V-3 and V-4). Ratios, ± s.d., represent 8 fibers treated with Carb and 10 treated with dTC.

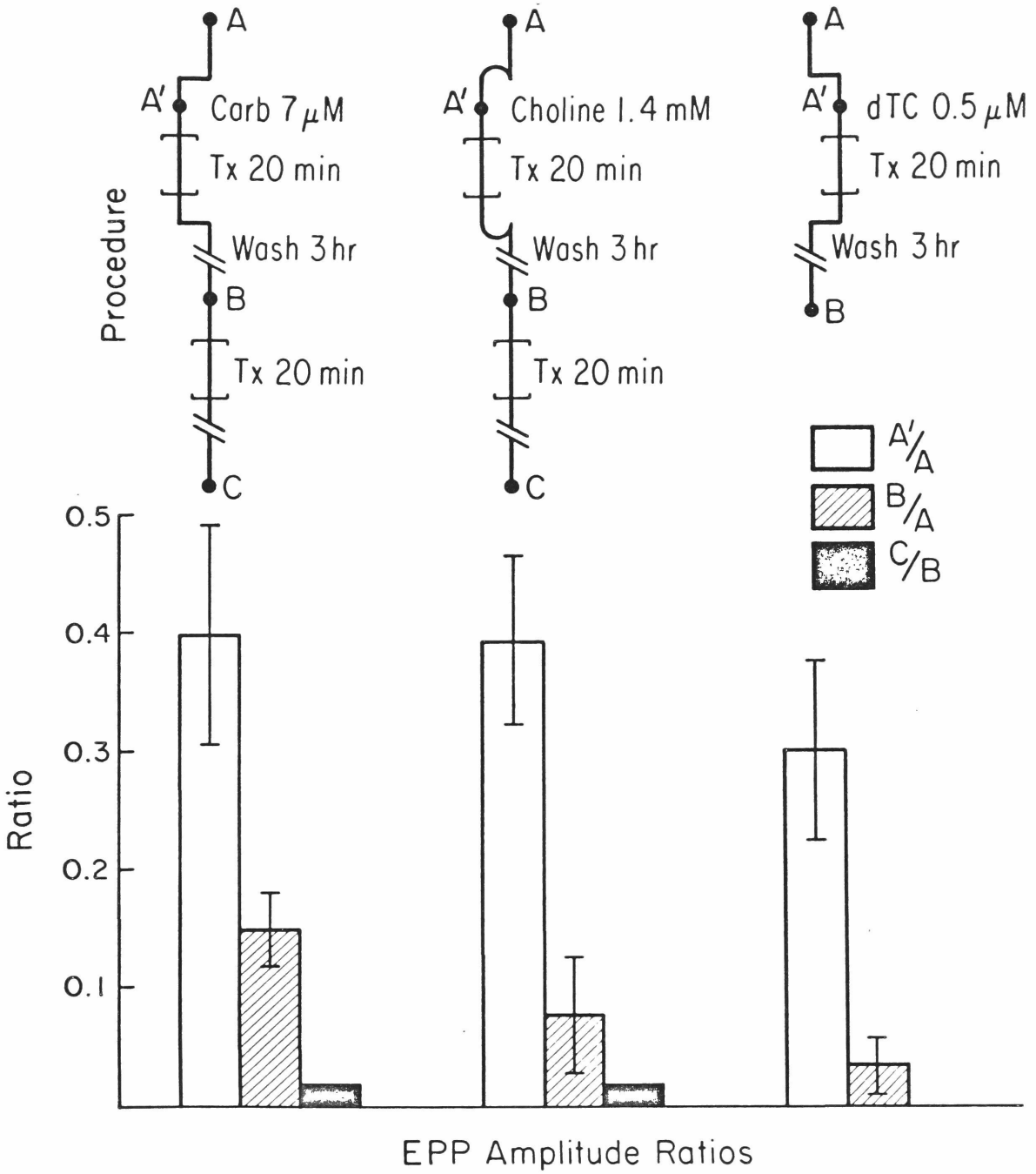


Figure V-6. EPP amplitude ratios, ± s.d., in each of 3 muscles treated with different reversible blocking agents. Five identified endplates in each muscle were sampled at A, A', B and C.

Nevertheless, the observation of unequal protection appears valid for the following reasons. Even if Carb decreased m, as suggested by the earlier experiments, then the actual protection of the receptors by Carb was even greater than observed, and the difference between the dTC protection and the Carb protection was even more marked than the EPP ratios in Figure V-6 indicate.

Choline acts partly as a depolarizing agent (like ACh and Carb) and partly as a nondepolarizing blocker (like dTC) (del Castillo and Katz, 1957c; Portela et al., 1970). One therefore expects its protective properties to lie somewhere between those of Carb and of dTC, as found experimentally.

Qualitative Differences in Protection

The next experiment (Fig. V-7) strongly supports the idea that Carb and dTC protect the receptors in qualitatively different fashions. Carbachol and dTC were used at concentrations which reduced the EPP to 3-5% of its control value. Although it would have been valuable to employ choline at doses of comparable potency, the necessary concentration (about 10 mM) also irreversibly blocked presynaptic ACh release.

Figure V-7 shows that α remains unchanged from its control value in the case of dTC block. This observation has been repeated under many conditions for this and lower dTC concentrations. In contrast to results obtained at higher dTC concentrations (Fig. V-4), these data indicate that dTC fails to protect ACh receptors against cobra toxin (see Discussion for a complete treatment of these results).

However, in the Carb case, the EPP no longer declines exponentially in time; α must be redefined in accordance with this interesting new phenomenon (see Discussion). In other preparations the run including Carb was taken before the control run, and the

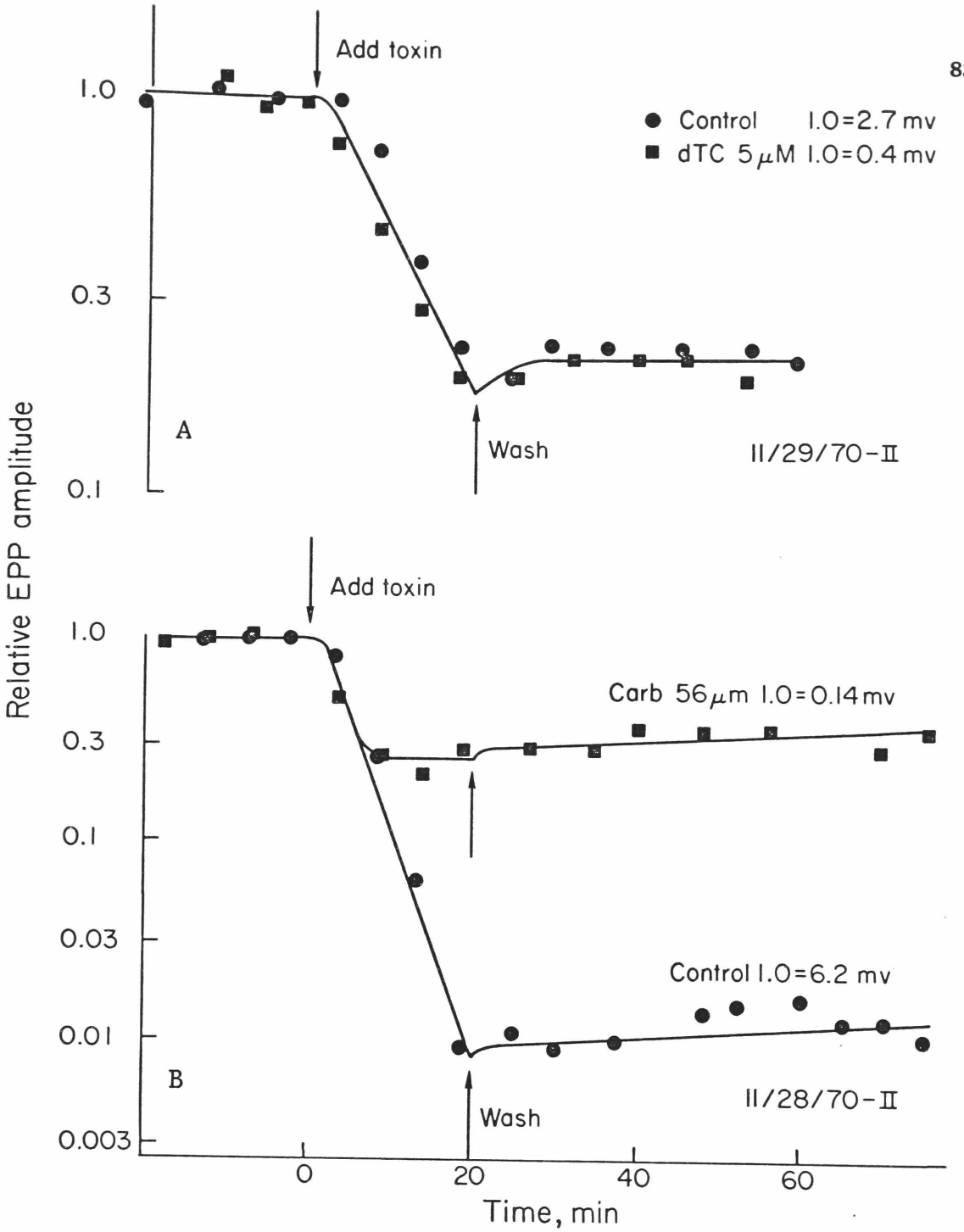


Figure V-7. Cobra toxin's effects on EPP during partial blockade by dTC (A) and Carb (B). A different muscle was used for A and B. ●, control run; only toxin was added to the Ringer and was present between the arrows. ■, test run; dTC or Carb was present before, during, and after toxin treatment.

toxin still caused the EPP to decline to a plateau in the presence of Carb. It is worthwhile to emphasize that this behavior seems not to represent a simple binding equilibrium, because the reversibility in the presence of Carb is no greater than in the control.

Discussion of the Protection Experiments

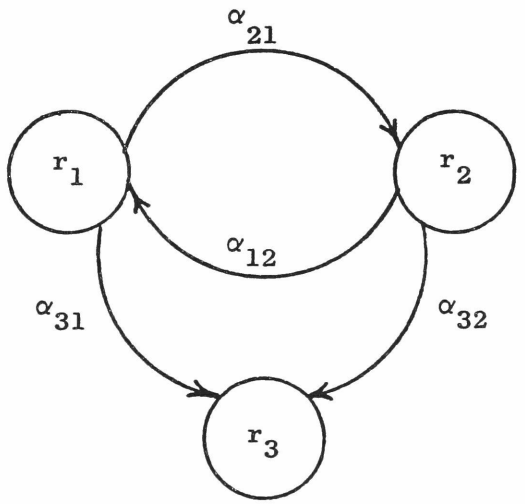
In the experiments described above, ACh potentials and EPPs were measured over a wide amplitude range in response to various drug and toxin treatments. It is our goal to interpret these data in terms of drug, toxin, and receptor action.

As a step toward building a model for these experiments, let us postulate different states of the receptor. These will be defined in terms of their availability to drugs and, ultimately, in terms of the ability to produce depolarizations. It would be exceedingly interesting if we knew how these different states correspond to molecular alterations in the receptor, perhaps arising from conformational changes or allosteric interactions. Chapter I gives a review of recent progress in this field; unhappily, our present knowledge limits us to operational definitions. Where contact can be made with other studies of receptor function, this will be noted.

The attack may be summarized before proceeding. We first present a general kinetic scheme (V-A) for interactions among the toxin, the receptor, and reversible agonists and antagonists. This scheme leads to differential equations (V-1). We immediately abandon the full formulation in order to understand its components. A lengthy discussion of curarization (Scheme V-B; Eqs. V-2 to V-3) and desensitization (Scheme V-C; Eqs. V-4 to V-5) contrasts the two processes and casts them into a form amenable to Scheme V-A and Eqs. V-1. We next discuss the toxin's action as a component of the scheme, essentially restating the conclusions of Chapter IV.

Reassembling the pieces, we define the protection parameter γ (Eq. V-8). First treating protection by dTC (Eqs. V-9 to V-12), we find that its action can be understood only by complicating our scheme (Scheme V-D). However, the protection by Carb is very well described by the original formulation (Eqs. V-13 to V-20), although the mathematics becomes cumbersome. In particular, the "plateau" behavior of Figure V-7 is explained as a decay with two time constants, and some quantitative statements about γ are made.

The Kinetic Scheme We define receptor state 1 as the one which produces depolarization (EPP or drug potentials) when presented with an agonist. We shall assume that r_1, the number of receptors in state 1, is proportional to the amplitude of EPPs (when we have controlled for quantal content) or of ACh or Carb potentials. The justifications for this assumption are presented in Chapter IV. State 2 is the inactive drug-receptor complex which reverts back to state 1 when the complex dissociates. State 3 is the inactive, irreversible toxin-receptor complex. Let r_i be the number of receptors in each state i, and α_{ji} be the rate constant for conversion from state i to state j. Representing the receptor states diagrammatically, we have:



V-A

Note that state 3, the irreversible toxin-receptor complex, can have no arrows leading from it. The constant called α in Equation IV-1b becomes α_{31} in this scheme.

This scheme leads to a set of first-order linear differential equations:

$$\dot{r}_1 = -(\alpha_{21} + \alpha_{31})r_1 + \alpha_{12} r_2, \qquad \text{V-1a}$$

$$\dot{r}_2 = \alpha_{21} r_1 - (\alpha_{12} + \alpha_{32})r_2, \qquad \text{V-1b}$$

and

$$\dot{r}_3 = \alpha_{31} r_1 + \alpha_{32} r_2. \qquad \text{V-1c}$$

It is illuminating to describe two simple cases.

Reversible Block Alone: Desensitization and Curarization First, in the absence of toxin, $\alpha_{32} = \alpha_{31} = 0$. If a reversible blocking agent, such as ACh, Carb, or dTC, is added to the system, and the initial condition is $r_1 = s$, $r_2 = 0$, a redistribution of receptors will occur. Since it is most convenient to measure r_1, the pertinent result is that

$$r_1 - \mu s = s(1-\mu)e^{-\lambda t}, \qquad \text{V-2a}$$

where

$$\mu = \frac{\alpha_{12}}{\alpha_{12} + \alpha_{21}}, \qquad \text{V-2b}$$

and

$$\lambda = \alpha_{12} + \alpha_{21}. \qquad \text{V-2c}$$

These equations imply that the system exponentially reaches a new equilibrium at $r_1 = \mu s$; the rate constant is $\lambda = \alpha_{12} + \alpha_{21}$.

In discussing this case, it should be stressed that the molecular interpretations for the states and rates α vary with the drugs involved. As mentioned, there are two large classes of reversible blockers, the agonists and the competitive antagonists (see Chapter I). The agonists, such as ACh and Carb, first produce depolarization, then desensitization.

Figure V-1 illustrates desensitization by Carb. The antagonists merely prevent depolarization.

It is thought that dTC is a "competitive" antagonist, because it binds reversibly with the ACh receptor site;

$$R + dTC \underset{k_{12}}{\overset{k_{21}}{\rightleftharpoons}} R \cdot dTC. \qquad \text{V-B}$$

Here we may identify R as state 1; R·dTC is state 2. It follows that

$$\alpha_{21} = k_{21} [dTC]; \qquad \text{V-3a}$$

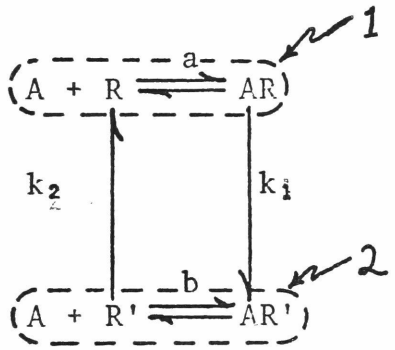
$$\alpha_{12} = k_{12}. \qquad \text{V-3b}$$

Jenkinson (1960) found that $k_{21}/k_{12} = 2.3 \times 10^6\ M^{-1}$ for dTC at the frog sartorius myoneural junction. This value is the "affinity constant." The determination, utilizing the "dose-ratio" method (Gaddum et al., 1955), assumed that only one dTC molecule combines with each receptor and that equal responses correspond to equal numbers of receptors occupied by the depolarizing drug (ACh or Carb). Jenkinson also discussed the implications of an ion-exchange reaction involving the drug and receptor, a concept first proposed by Ing and Wright (1933) and explored recently in connection with both depolarization and intracellular uptake of cholinergic drugs (Taylor and Nedergaard, 1965; Taylor et al., 1970).

For the case of dTC, attempts have been made to measure k_{21} and k_{12} either by the method of micro-ionophoretic application (del Castillo and Katz, 1957a; Waud, 1967) or by rapid changes (< 10 sec) of the perfusion medium (Goldsmith, 1963; Waud, 1967). Waud in particular has presented arguments, based on solutions to the diffusion equations, suggesting that α_{21} is so large that access to the receptors limits the onset of dTC block. Although no quantitative limits are given, it would appear that $1/\alpha_{21}$ is less than 100 msec for doses of dTC which give $\mu \leq 0.5$ --i.e., doses which block more than half the receptors (see Eq. V-2).

This situation, of rapid transitions between receptor states 1 and 2, contrasts sharply with the desensitization process. Katz and Thesleff (1957) studied desensitization by applying ACh and Carb micro-ionophoretically from one barrel of a double pipette, while monitoring transient ACh or Carb depolarizations caused by pulses from the other barrel. The procedure was directly comparable to micro-ionophoretic application of dTC (del Castillo and Katz, 1957a), but the time course for the onset of desensitization was many times longer than for curarization. An additional difference was that recovery from desensitization, upon cessation of the steady drug release, occurred with about the same time course as onset. With dTC, on the other hand, recovery is longer than onset, as predicted by scheme V-B and Eqs. V-3.

Katz and Thesleff (1957) developed a model which explained these characteristics of desensitization. They postulated a cycle of receptor states. If A is an agonist molecule,



V-C

The affinity constants a and b govern equilibria which occur within times short compared with diffusion--or, for our purposes, instantaneously.

$$a = \frac{[A \cdot R]}{[A][R]} \; ; \quad \text{V-4a} \qquad b = \frac{[A \cdot R']}{[A][R']} . \quad \text{V-4b}$$

R is the free receptor; A·R is the active complex which produces depolarization. The desensitized complex A·R' is incapable of producing depolarization; R' is the free, desensitized receptor. The two rate constants K_1 and K_2 are of the order of fractions of inverse seconds.

Some comments on this model are pertinent. The first is that the cycle as written requires an energy input. Katz and Thesleff (1957) considered writing each step as reversible; in that case, the rate constants lose their independence. After we transform this cycle into the notation of Scheme V-A, the reader will see that this distinction will lose its formal, if not its molecular, significance. Second, Katz and Thesleff estimated b/a is about 20, so that appreciable desensitization could occur without substantial depolarization. Finally, the bottom reaction in Scheme V-C confers the property of rapid recovery. We shall not consider this feature in our analysis, because my experiments do not deal with the time course of recovery.

In Scheme V-C, two compartments 1 and 2 have been constructed with dashed lines. These correspond, for the purpose of our analysis, with states 1 and 2 in Scheme V-A. Referring to Eq. V-4, the following relations hold:

$$\alpha_{21} = \frac{k_1 a[A]}{1 + a[A]} ; \quad \text{V-5a}$$

$$\alpha_{12} = \frac{k_2}{1 + b[A]} . \quad \text{V-5b}$$

Summarizing our progress since presenting Scheme V-A, we have cast both the curarization and desensitization processes into the same formal mechanism. Both may now be described by Eqs. V-2, which are simplifications of the general Eqs. V-1. We have also seen how the

formal Scheme V-A relates to known features of the two processes. In this light, the difference between desensitization and curarization is a quantitative one. For a given μ, the rate constants α_{21}, α_{12} and λ for desensitization are much smaller--perhaps by a factor of 200--than for curarization. This is strikingly evident if one compares the onset of desensitization in the experiments of Katz and Thesleff (1957) with the onset of dTC action as seen by del Castillo and Katz (1957a).

Other investigators, primarily using bath-applied Carb, have confirmed the long time course of desensitization. Nastuk and his collaborators term the process "receptor inactivation" but conclude, in constrast to Waud (1968), that this is the same phenomenon called "desensitization" by Thesleff (1955). In general, λ in the bath-applied Carb case is less than with micro-ionophoretic application, but this difference is ascribed to the fact that ionophoretic pulses produce a very high local concentration of drug (Nastuk, 1967).

Nastuk and his collaborators studied the effects on desensitization of Carb concentrations, tonicity of the bathing solution, divalent cations, and denervation (Manthey, 1966, 1970; Nastuk and Gissen, 1965; Nastuk and Parsons, 1970). They were impressed that divalent cations, particularly Ca^{++} and UO_2^{++}, produce striking increases in λ for desensitization (Eq. V-2). They therefore proposed (Nastuk, 1967) that a phosphate-containing macromolecule is bound by Ca^{++} to the anionic site of a protein, possibly acetylcholinesterase. Quaternary ammonium ions compete with Ca^{++} for the phosphoryl group. Desensitization occurs when Ca^{++}, possibly from intracellular sources (Nastuk and Parsons, 1970), displaces the drug from the anionic site to another, inactive site. Alternatively, the trivalent phosphate group could be capable of binding several drug molecules; depolarization would occur if and only if one drug molecule is bound (Nastuk, 1967). This mechanisms would explain the increase in λ with increasing drug concentration. Nastuk and Parsons (1970) also consider the possibility that desensitization occurs when free intracellular Ca^{++} binds with

anionic sites on the inner surface of the postsynaptic membrane, somehow resulting in a reduced ionic permeability. Manthey (1970) offers further evidence for this mechanism based on the time required--about 13 sec--for changes in extracellular Ca^{++} to cause changes in the rate of desensitization.

Additional evidence for Ca^{++} interference with ionic permeability arises from experiments with micro-ionophoretic ACh application (Magazanik and Vyskočil, 1970). Polyvalent ions were found to increase the rate of desensitization in the same order that they bind to phospholipids as well as that in which they affect certain other nerve membrane resistances. Magazanik and Vyskočil emphasize that this mechanism for desensitization extends beyond the receptor's "recognition" function to the "permeation" function (Chapter I). This in turn implies that desensitization involves a more profound change in receptor structure than does curarization--a conclusion which will be supported by experiments.

These mechanisms, while different from the one proposed by del Castillo and Katz, may be treated with Scheme V-A and would therefore result in the kinetics and equilibria described by Eq. V-2.

Toxin Acting Alone. The second simple case is essentially the experiments reported in Chapters III and IV: the toxin irreversibly inactivates receptors; no reversible blocking agent is present ($\alpha_{21} = 0$; $r_2 = 0$). All the receptors eventually go to state 3 with kinetics described by:

$$r_1 = se^{-\alpha_{31}t} = s-r_3, \qquad \text{V-6}$$

where s is again the original number of receptors. This is the familiar exponential decline in response amplitude represented by Figs. III-1, III-2, III-3, and IV-1.

In Chapter IV, the rate of toxin action is analyzed. The conclusion is that the initial stages of toxin action are limited by

diffusion of toxin molecules to the vicinity of the receptors; the toxin concentration near the receptors eventually equals that in the external bath. At this point the amount of toxin near the receptor is probably not perturbed by interactions with the receptors or other muscle components. Pseudo-first order kinetics then prevail, as described by Eq. V-6. Referring to Eq. IV-1b, we have

$$\alpha_{31} = k_T[T], \qquad \text{V-7}$$

where [T] is toxin concentration and k_T is experimentally equal to 5.5×10^{-4} sec^{-1} liter $mole^{-1}$.

Combined dTC Block and Toxin Treatment We are now in a position to discuss the results, presented in this chapter, of experiments with both toxin and reversible blockers of the receptor.

We first consider the case of dTC as the reversible agent. The experiments lead to the following conclusions: at high dTC concentrations, receptors are partially protected against the toxin. This is evident from Figure V-4, in which the dTC concentration was 26 μM. As defined by Eq. V-2a, μ was 0.004. At lower dTC concentrations, there was no measurable protection. This is evident from Figure V-6 ([dTC] = 0.5 μM; μ = 0.3) and Figure V-7 ([dTC] = 5 μM; μ = 0.03).

These results cannot be readily interpreted in terms of the general scheme V-A. The question posed by the dTC protection experiment is, can the toxin irreversibly inactivate the curarized (state 2) as well as the free (state 1) receptor? That is, how does α_{32} compare with α_{31}? We may parametrize the amount of protection by taking

$$\alpha_{32} = \gamma\alpha_{31} \qquad \text{V-8}$$

The experiments at μ = 0.004 indicate that $\gamma < 1$, but at μ = 0.3 and 0.03, γ is approximately one. Scheme V-A, as we have discussed it, allows for no such influence of μ on γ.

Before modifying V-A to fit these observations, it is important to note how the kinetics of toxin action during reversible block, as described by Figure V-7, support the conclusion of no protection at low dTC concentrations. As we have discussed, $1/\alpha_{12}$ and $1/\alpha_{21}$ for dTC are 0.1 sec or less, depending on the dTC concentration. On the other hand, $1/\alpha_{31}$ for the toxin's action is several minutes.

Under these conditions, the transitions between states 1 and 2 may be treated as instantaneous. Eqs. V-1 lead immediately to simple solutions:

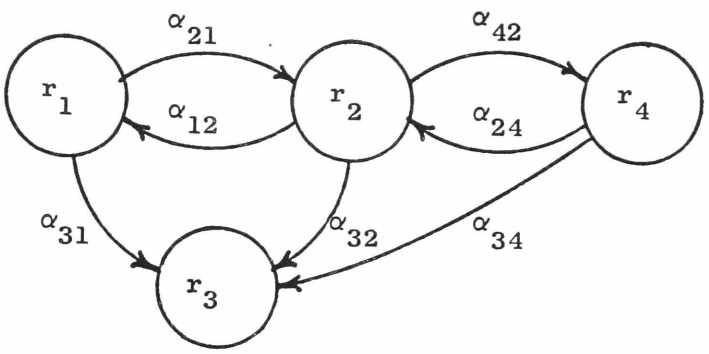
$$-\dot{r}_1 = [\mu + \gamma(1-\mu)]\alpha_{31} r_1, \qquad \text{V-9}$$

$$r_1 = \mu s e^{-[\mu + \gamma(1-\mu)]\alpha_{31} t}. \qquad \text{V-10}$$

Since μ is smaller than 1, protection is observed during reversible block as a decrease in the observed rate constant for receptor inactivation. Complete protection ($\gamma = 0$) would decrease the rate constant to the fraction μ. No decrease showed in the experiments at $\mu = 0.03$ (Fig. V-7), nor was any observed for higher values of μ.

Since protection occurred at $\mu = 0.004$ (high concentration of dTC), one would expect to find a decrease in the rate constant for receptor inactivation under these conditions. However, the high dTC concentration also meant that the curarized EPP was too small for reliable kinetic measurements. I had to resort to the experiments of Figure V-4, which involved EPP measurements before and after, but not during, dTC treatment.

The simplest way to explain the concentration-dependent protection by dTC is by an addition to Scheme V-A.



V - D

We suppose that dTC creates an additional curarized state, which we label 4. This state may be formed when a second dTC molecule binds to state 2, whose characteristics have been described. By analogy with Eq. V-3a, we suppose that

$$\alpha_{42} = k_{42} [dTC], \qquad \text{V-11}$$

where k_{42} is a second-order rate constant. By analogy with Eq. V-3b, α_{24} is a first-order rate constant. As in the case of α_{21} and α_{12}, we suppose that α_{24} and α_{42} are large enough so that equilibration between states 2 and 4 occurs within fractions of a second, although this point is neither crucial to the discussion nor amenable to experimentation. At equilibrium,

$$r_4 = r_2 \frac{k_{42}}{\alpha_{24}} [dTC], \qquad \text{V-12}$$

and the important point is that the fraction of curarized receptors in state 4 increases with [dTC]. If state 4 is resistant to the toxin, this means that $\alpha_{34} < \alpha_{31}$. This scheme explains how dTC can appear to give protection against the toxin only at higher concentrations.

Is state 4 really an alteration in the receptor produced only by high concentrations of dTC and other competitive inhibitors, or is it a major change in the postsynaptic membrane which can arise from the binding of a large class of molecules? I have little data bearing on this question, but it should be noted that choline, another quaternary ammonium compound, produced only slight protection (Fig. V-6) at concentrations 50 times greater than the dTC concentrations in Figure V-4. In connection with this point, it would be interesting to measure the protection afforded by other strychnos alkaloids which are not cholinergic blocking agents (Taylor and Nedergaard, 1965).

Combined Carb Block and Toxin Treatment In contrast to the fitful protection provided by dTC, Carb protected the receptor against cobra toxin at all concentrations tested, from 7 μM ($\mu = 0.4$) to 140 μM ($\mu = 0.005$).

The kinetics shown in the lower part of Figure V-7 are an interesting aspect of the toxin's action during Carb protection. Scheme V-A can explain this data. We begin by reviewing the experimental procedure. Before addition of toxin, the preparation has been desensitized by a steady concentration of Carb. This situation is described by Eqs. V-2. If the original number of receptors was s, the new size of r_1 is μs.

When toxin is added in the presence of Carb, Equations V-1 hold. The general solution in terms of the measurable quantity, r_1, is

$$r_1 = \beta_+ e^{-\lambda_+ t} + \beta_- e^{-\lambda_- t}, \qquad \text{V-13}$$

where

$$\lambda_\pm = \tfrac{1}{2}[\alpha_{21} + \alpha_{12} + \alpha_{31} + \alpha_{32} \pm Q], \qquad \text{V-14}$$

and

$$Q = [(\alpha_{21} + \alpha_{12} + \alpha_{31} + \alpha_{32})^2 - 4(\alpha_{12}\alpha_{31} + \alpha_{21}\alpha_{32} + \alpha_{31}\alpha_{32})]^{\frac{1}{2}}.$$

The values for $\beta_{\pm}$ may be determined by noting that, for t = 0,

$$\beta_+ + \beta_- = \mu s. \qquad \text{V-15a}$$

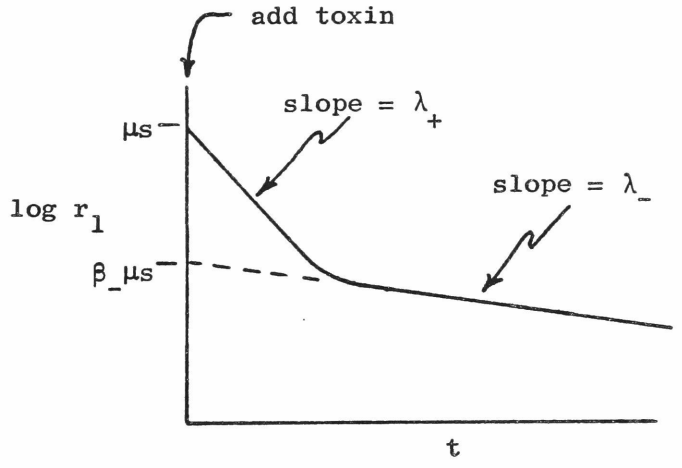
Also, from Eq. V-1a, at t = 0,

$$\lambda_+\beta_+ + \lambda_-\beta_- = (\alpha_{21} + \alpha_{12})\mu s - \alpha_{12}(1-\mu)s. \qquad \text{V-15b}$$

The simultaneous linear equations V-15 solve as follows:

$$\beta_{\pm} = \frac{\mu s}{2}\left[1 \pm \frac{\alpha_{31} - \alpha_{21} - \alpha_{12} - \alpha_{32}}{Q}\right]. \qquad \text{V-16}$$

Thus, Eqs. V-1 predict an exponential decline to zero with two time constants. On semilogarithmic coordinates, the solution plots as two linear segments:



This plot has the same shape as the experimental run in Figure V-7. A brief argument will be given here to show that the quantitative features of Figure V-7 can be predicted from Equations V-13 to V-16 and reasonable values for α_{12}, α_{21}, α_{31}, and α_{32}. First, Equations V-2b and V-2c may be used for a determination of α_{12}, α_{21}, if we have a value for λ for desensitization alone.

There are no experiments in the literature with $[Ca^{++}] = 2$ M and [Carb] = 56 μM, as in Figure V-7, but Manthey (1970) reports a half time ($= \frac{0.693}{\lambda}$) of 55 sec for $[Ca^{++}] = 1.8$ mM and [Carb] = 137 μM. This result was obtained in potassium-depolarized fibers, and the transformation to normal resting potential is uncertain (Magazanik and Vyskočil, 1970)]. Manthey's results and those of Katz and Thesleff (see Eq. V-7a) indicate that λ increases less than linearly with [Carb]. I therefore estimate that the half-time for desensitization in my experiments was 100 sec, giving $\lambda = 7 \times 10^{-3}$ sec^{-1}.

As mentioned, μ in my experiments was 0.03. Therefore, from Equations V-2b, V-2c,

$$\alpha_{12} = 2.3 \times 10^{-4} \text{ sec}^{-1}$$

$$\alpha_{21} = 6.8 \times 10^{-3} \text{ sec}^{-1}$$

Next, the value for α_{31} is just the slope of the control run in the lower part of Figure V-7b, or 3.3×10^{-3} sec^{-1}.

Finally, α_{32} represents the vulnerability of the desensitized receptor to the toxin and is therefore a most interesting parameter. The data in Figures V-3, V-5, V-6 and V-7 all suggest that $\alpha_{32} < \alpha_{31}$. As in the discussion of dTC protection (Eq. V-8), we let $\alpha_{32} = \gamma\alpha_{31}$. A decay with two time constants occurs only for $\gamma < 1$, which corresponds to protection. Predicted and observed values of $\lambda_{\pm}$ and $\beta_{\pm}$ are given in Table III as a function of γ.

Considering the uncertainties in α_{12}, α_{21} and the fact that Equation V-16 involves strong cancellation among the values for α, the agreement between theory and experiment is good. The chief effect of increasing γ is to increase λ_-, the slope of the slow decay. The data seem inconsistent with γ greater than 0.1. Therefore, protection seems nearly complete. However, this kinetic data still leaves open the possibility that desensitized receptors retain a small vulnerability to the toxin.

The experiments in Figures V-3, V-5, V-6, approach this point by utilizing the fact that states 2 and 3 are distinguished from each

Table III

Kinetics of receptor inactivation by toxin during Carb treatment, according to experiment and as predicted by Scheme V-A and Equations V-1, V-12, and V-14. See text for a discussion of assumptions and parameter γ.

	λ_+, sec^{-1} ($x10^3$)	λ_-, sec^{-1} ($x10^3$)	β_+ / μs	β_- / μs	Q, sec^{-1} ($x10^3$)
Experiment (Fig. V-7)	> 5	< 0.5	0.75	0.25	
Predicted: $\gamma = 0$	10.6	0.2	0.30	0.70	10.35
$\gamma = 0.1$	10.8	0.7	0.27	0.73	10.1
$\gamma = 0.5$	10.6	1.8	0.17	0.83	8.9

other on the basis of reversibility. Upon washing out the agonist, all the receptors in state 2 should revert to state 1, and r_3 may be determined by subtraction from s. In general, Equations V-1 solve for r_3 in terms of the same λ_+, λ_-. Remembering that $r_3 = 0$, $r_1 = \mu s$, and $r_2 = (1-\mu)s$ at $t = 0$, and $r_3 = s$ at $t = \infty$, we have

$$s - r_3 = \kappa_+ e^{-\lambda_+ t} + \kappa_- e^{-\lambda_- t} . \qquad \text{V-17}$$

The initial conditions give

$$\kappa_+ + \kappa_- = s, \qquad \text{V-18a}$$

and, from Eq. V-1c,

$$\lambda_+ \kappa_+ + \lambda_- \kappa_- = \alpha_{31}\mu s + \alpha_{32}(1-\mu)s. \qquad \text{V-18b}$$

Equations V-18 solve for

$$\kappa_\pm = \frac{s}{2}\left[1 \mp \frac{(1-2\mu)(\alpha_{31} - \alpha_{32}) + \alpha_{12} + \alpha_{21}}{Q}\right]. \qquad \text{V-19}$$

Figures V-3 and V-5 illustrate the difficulties in actual application of Equations V-17, V-18, and V-19. The recovery from treatment with large Carb concentration is erratic and may involve permanent changes in quantal content. If one looks at protection of MEPP instead of EPP amplitudes, in order to eliminate presynaptic effects, Figure V-5 suggests that desensitized receptors received nearly complete protection. We therefore adopt the value $\frac{s - r_3}{s} = 0.9$. From Equation V-7, $\alpha_{31} = 6.05 \times 10^{-3}$ sec^{-1}. We again utilize Manthey's (1966, 1970) measurements on the kinetics of desensitization to estimate $\lambda = 1.26 \times 10^{-2}$ sec^{-1} (Eq. V-2). In these experiments, μ was too small to measure; we adopt the upper limit, $\mu = 0.005$. Then, by Equation V-2c

$$\alpha_{12} = 5.3 \times 10^{-5} \text{ sec}^{-1}$$

$$\alpha_{21} = 1.2 \times 10^{-2} \text{ sec}^{-1}.$$

We next determine α_{12}, α_{21} for the experiment of Figure V-6. No studies appear in the literature for the time course of desensitization with Carb concentrations as low as these (7 μM). On the basis of preliminary data, I estimate $\lambda = 3 \times 10^{-3}$ sec^{-1}. For $\mu = 0.4$, Equations V-2 give

$$\alpha_{12} = 1.2 \times 10^{-3} \text{ sec}^{-1},$$

$$\alpha_{21} = 1.8 \times 10^{-3} \text{ sec}^{-1}.$$

No permanent effect on quantal content was noticeable in this experiment.

Table IV compares results of these two experiments with predicated values for $\frac{s-r_3}{s}$ on the basis of a variable γ and Equations V-17, V-18, and V-19. Again the data are fit well by $\gamma = 0.1$ or less. Like the kinetic data, these experiments are consistent with nearly complete protection in the desensitized state.

In the future, it may prove possible to identify γ with the fine structure of state 2. For instance, referring to Scheme V-C, R' might be as vulnerable to the toxin as R, but AR and AR' completely resistant. By Equations V-4 and V-5, this leads to

$$\gamma = \frac{[R']/([R'] + [AR'])}{[R]/([R] + [AR])} = \frac{1 + a\,[A]}{1 + b\,[A]}. \qquad \text{V-20}$$

We have little basis on which to explore this model in detail. But it does predict that γ will approach the lower limit, $\frac{a}{b}$, for large values of A. It also allows an estimate of a, the Carb affinity of the ACh receptor. Taking $\frac{a}{b} = 0.05$ (Katz and Thesleff, 1957) and $\gamma = 0.1$ at [Carb] = 7 μM (Table IV), Equation V-20 solves for $a = 1.3 \times 10^5$ M^{-1}. This value agrees with the results of other work (Fatt, 1950; del Castillo and Katz, 1957b. For a rigorous discussion of methods for determining a, see Furchgott, 1964; and Waud, 1968).

Table IV

Carb protection against cobra toxin, 1.1×10^{-7} M. Details of the calculations and assumptions are given in the text.

See Fig.	[Carb]	μ	$\frac{s-r_3}{s}$ observed	$\frac{s-r_3}{s}$ predicted	γ
V-3,V-5	140μM	5×10^{-3}	0.9		
				0.99	0
				0.82	0.1
V-6	7μM	0.4	0.15		
				0.27	0
				0.17	0.1
				0.06	0.2

Conclusions

These experiments involved micro-ionophoretic applications of agonists (ACh and Carb) and bath application of an agonist (Carb) and a competitive antagonist (dTC). It was shown that focal application of agonists provides focal protection against cobra toxin. According to a simple model (V-A), the bath-applied agonists also provided nearly complete protection ($\gamma < 0.1$) in the desensitized state. Furthermore, it was shown that the model accounts for interesting two-stage kinetics of toxin action (Fig. V-7) when one takes account of the slow rate constants for entering and leaving the desensitized state.

For the competitive inhibitor, dTC, the model requires modification (Scheme V-D) so that protection occurs only when high concentrations of dTC produce a new receptor state.

The experiments therefore imply that agonists produce a rather more profound change in receptor structure than do competitive antagonists. Current concepts of drug action would seem to agree. Of some relevance are the conclusions of Nastuk and Parsons (1970), Manthey (1970), and Magazanik and Vyskočil (1970), that desensitization interferes with the "permeation" function of the receptor in addition to the "recognition" function (Chapter I, pp. 2 - 6). Likewise, Changeux and Podleski (1968) found that agonists, but not competitive antagonists, produce changes in the co-operative properties of the postsynaptic membrane.

The present model (V-A) sheds some light on the "metaphilic" reagents (Rang and Ritter, 1969). Their action has been interpreted as arising from a greater reactivity for the desensitized than for the normal receptor. In terms of Scheme V-A and Equation V-8, we may say that, for metaphilic reagents, $\gamma > 1$.

The present analysis also shares features with that of Steinbach (1968a, b), who studied processes with rate constants several

orders of magnitude faster than the ones I have considered. Steinbach found that Xylocaine and its derivatives alter the time course of EPPs and ACh potentials by reducing the early phase of the endplate current and prolonging its later phase. Like the effects I have described, this behavior was shown to arise from differences in a drug's (in my case, a toxin's) affinity for active and inactive receptors.

CHAPTER VI

CONCLUSIONS

I initiated this study in order to establish cobra toxin as a comprehensible pharmacological tool for investigations of cholinergic transmission. My work fell naturally into three major divisions. First, I showed that the toxin's action is confined to the postsynaptic nicotinic acetylcholine (ACh) receptor (Chapter III). Second, I showed that this action probably involves an irreversible monomolecular binding to the receptor (Chapters IV and V). Finally, I have begun to use the toxin as a probe for distinguishing various states of the receptor (Chapter V).

One important hope underlying my investigation was that we could understand these toxins well enough to employ them as biochemical markers for isolating the ACh receptor. This hope has recently received encouragement from studies involving α-bungarotoxin, the closely related toxin from the Formosan krait (see Table I). One group (Changeux et al., 1970) has followed the binding of pharmacological agents to a partially purified protein which fulfills many criteria for the receptor. Bungarotoxin inhibits this binding in the same fashion one would predict from the electrophysiological results.

Another group (Miledi et al., 1971) has also described experiments which led to a partial purification of the receptor. In this case, a bound ^{131}I -α-bungarotoxin label was followed through isolation procedures. These investigators also briefly report results which corroborate my finding, that desensitization protects the receptor against the toxin more completely than does curarization.

One expects that such studies will soon bear fruit in two areas. First, with the isolated receptor in hand, we shall be able to investigate the molecular mechanism by which ACh induces ionic permeability changes in postsynaptic structures. Second, we shall have the proper tool to study the biology of synaptic plasticity and development.

BIBLIOGRAPHY

Andersen, P., and J.K.S. Jansen (eds.) (1970). Excitatory Synaptic Mechanisms. Universitets Forlaget, Oslo.

Baker, R.W., C.H. Chothia, P. Pauling, and T.J. Petcher (1971). Structure and activity of muscarinic stimulants. Nature 230, 439-445.

Barlow, R.B., and H.R. Ing (1948). Curare-like action of bis-quaternary ammonium salts. Brit. J. Pharmacol. 3, 298-304.

Beers, W.H., and E. Reich (1970). Structure and activity of acetylcholine. Nature 229, 917-922.

Bennett, M.V.L. (1961). Modes of operation of electric organs. Ann. N.Y. Acad. Sci. 94, 458-509.

Bennett, M.V.L. (1970). Comparative physiology: electric organs. Ann. Rev. Physiol. 32, 471-528.

Bennett, M.V.L. (1971). Analysis of parallel excitatory and inhibitory synaptic channels. J. Neurophysiol. 34, 69-75.

Bennett, M.V.L., M. Wurzel, and H. Grundfest (1961). The electrophysiology of electric organs of marine electric fishes. I. Properties of the electroplaques of Torpedo nobiliana. J. Gen. Physiol. 44, 757-818.

Birks, R., H.E. Huxley, and B. Katz (1960). The fine structure of the neuromuscular junction of the frog. J. Physiol. 150, 134-144.

Blankenship, J.E., H. Wachtel, and E.R. Kandel (1971). Ionic mechanisms of excitatory, inhibitory, and dual synaptic actions mediated by an identified interneuron in abdominal ganglion of Aplysia. J. Neurophysiol. 34, 76-92.

Botes, D.P. (1970). Purification and amino acid sequence of three neurotoxins from the Cape cobra (Naja nivea). Toxicon 8, 125-126.

Botes, D.P., and D.J. Strydom (1969). A neurotoxin, Toxin α, from Egyption cobra (Naja haje haje) venom. I. Purification, properties, and complete amino acid sequence. J. Biol. Chem. 244, 4147-4157.

Brock, L.G., and R.M. Eccles (1958). The membrane potentials during rest and activity of the ray electroplate. J. Physiol. 142, 251-274.

Brock, L.G., R.M. Eccles, and R.D. Keynes (1953). The discharge of individual electroplates in Raja clavata. J. Physiol. 122, 4P.

Brown, A.M. (1967). Cardiac sympathetic adrenergic pathways in which synaptic transmission is blocked by atropine sulphate. J. Physiol. 191, 271-288.

Burgen, A.S.V. (1966). The drug-receptor complex. J. Pharmacol. (London) 18, 137-149.

Burn, J.H. (1963). The release of norepinephrine from the sympathetic postganglionic fiber. Bull. Johns Hopkins Hosp. 112, 167-182.

Carey, E.J., and E.A. Wright (1961). The site of action of the venom of the sea-snake Enhydrina schistosa. Trans. Roy. Soc. Trop. Med. Hyg. 55, 153-160.

Castillo, J. del, and B. Katz (1954a). Quantal components of the endplate potential. J. Physiol. 124, 560-573.

Castillo, J. del, and B. Katz (1954b). The membrane change produced by neuromuscular transmitter. J. Physiol. 125, 546-565.

Castillo, J. del, and B. Katz (1955). On the localization of acetylcholine receptors. J. Physiol. 128, 157-181.

Castillo, J. del, and B. Katz (1965a). Localization of active spots within the neuromuscular junction of the frog. J. Physiol. 132, 630-649.

Castillo, J. del, and B. Katz (1965b). Biophysical aspects of neuromuscular transmission. Progr. Biophys. Biophys. Chem. 6, 121-170.

Castillo, J. del, and B. Katz (1957a). A study of curare action with an electrical micro-method. Proc. Roy. Soc. B 146, 339-356.

Castillo, J. del, and B. Katz (1957b). A comparison of acetylcholine and stable depolarizing agents. Proc. Roy. Soc. B 146, 363-368.

Castillo, J. del, and B. Katz (1957c). Interaction at endplate receptors between different choline derivatives. Proc. Roy. Soc. B 146, 369-381.

Chang, C.C. (1960). Studies on the mechanism of curare-like action of Bungarus multicinctus venom. II. Effect on response of rectus abdominus muscle of the frog to acetylcholine. J. Formosan Med. Assoc. 59, 315-333.

Chang, C.C., and C.Y. Lee (1963). Isolation of neurotoxins from the venom of Bungarus multicinctus and their modes of neuromuscular blocking action. Arch. Int. Pharmacodyn. 144, 241-257.

Chang, C.C., and C.Y. Lee (1966). Electrophysiological study of neuromuscular blocking action of cobra neurotoxin. Brit. J. Pharmacol. 28, 172-181.

Changeux, J.-P., M. Kasai, and C.Y. Lee (1970). Use of a snake venom toxin to characterize the cholinergic receptor protein. Proc. Nat. Acad. Sci. U.S.A. 67, 1241-1247.

Changeux, J.-P., and T.R. Podleski (1968). On the excitability and cooperativity of the electroplax membrane. Proc. Nat. Acad. Sci. U.S.A. 59, 944-950.

Chiarandini, D.J., and H.M. Gerschenfeld (1967). Ionic mechanism of cholinergic inhibition in molluscan neurons. Science 156, 1595-1596.

Chiarandini, D.J., E. Stefaini, and H.M. Gerschenfeld (1967). Ionic mechanisms of cholinergic excitation in molluscan neurons. Science 156, 1597-1599.

Chothia, C.L., and P. Pauling (1970). The conformation of cholinergic molecules at nicotinic nerve receptors. Proc. Nat. Acad. Sci. U.S.A. 65, 477-482.

Chou, T.C., and C.Y. Lee (1969). Effect of whole and fractionated cobra venom on sympathetic ganglionic transmission. Europ. J. Pharmacol. 8, 326-330.

Ciani, S., and Edwards, C. (1963). The effect of acetylcholine on neuromuscular transmission in the frog. J. Pharmacol. Exp. Ther. 142, 21-23.

Cole, W.V. (1955). Motor endings in the striated muscle of vertebrates. J. Comp. Neurol. 102, 671-716.

Colomo, F., and R. Rahamimoff (1968). Interaction between sodium and calcium ions in the process of transmitter release at the neuromuscular junction. J. Physiol. 198, 202-218.

Couteaux, R. (1947). Contribution a l'étude de la synapse myoneurale. Rev. Canad. Biol. 6, 563-711.

Curtis, D.R., and Rosamund M. Eccles (1958). The effect of diffusional barriers upon the pharmacology of cells within the central nervous system. J. Physiol. 141, 446-463.

Dale, H.H. (1914). The action of certain esters and ethers of choline, and their relation to muscarine. J. Pharmacol. Exp. Therap. 6, 147-190.

Dale, H.H. (1937). Transmission of nervous effects by acetylcholine. Harvey Lect. 32, 229-245.

Danielli, J.F., J.F. Moran, and D.J. Triggle (eds) (1970). Fundamental concepts in drug-receptor interactions. Academic Press, New York.

Davson, H. (1957). Physiology of the ocular and cerebrospinal fluids. Churchill, London.

Eaker, D., and J. Porath (1967). The amino acid sequence of a neurotoxin from Naja nigricollis venom. Japan J. Microbiol. 11, 353-355.

Eccles, J.C. (1964). The Physiology of Synapses. Academic Press, New York.

Eccles, J.C., Rosamund M. Eccles, and P. Fatt (1958). Pharmacological investigations on a central synapse operated by acetylcholine. J. Physiol. 131, 154-169.

Eccles, J.C., P. Fatt, and K. Koketsu (1954). Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. J. Physiol. 126, 524-562.

Falk, G., and Fatt, P. (1965). Electrical impedance of striated muscle and its relation to contraction. In: Studies in Physiology. D.R. Curtis and A.K. McIntyre, eds. Berlin: Springer Verlag, Berlin.

Fatt, P. (1950). The electromotive action of acetylcholine at the motor endplate. J. Physiol. 111, 408-422.

Fatt, P., and B. Katz (1951). An analysis of the endplate potential recorded with an intracellular electrode. J. Physiol. 115, 320-370.

Flacke, W., and T.S. Yeoh (1968a). The action of some cholinergic agonists and anticholinesterase agents on the dorsal muscle of the leech. Br. J. Pharmacol. Chemother. 33, 145-153.

Flacke, W., and T.S. Yeoh (1968b). Differentiation of acetylcholine and succinylcholine receptors in leech muscle. Br. J. Pharmacol. Chemother. 33, 154-161.

Frank, K., and L. Tauc (1964). Voltage-clamp studies of molluscan neuron membrane properties. In: The Cellular Functions of Membrane Transport. J. Hoffman, ed. Prentice-Hall, Englewood Cliffs, N.J.

Frazier, W.T., E.R. Kandel, I. Kupferman, R. Waziri, and R.E. Coggeshall (1967). Morphological and functional properties of identified neurons in the abdominal ganglion of Aplysia californica. J. Neurophysiol., 30, 1288-1351.

Furchgott, R.F. (1964). Receptor mechanisms. Ann. Rev. Pharmacol. 4, 21-50.

Gaddum, J.H., K.A. Jameed, D.E. Hathway, and F.F. Stevens (1955). Quantitative studies of antagonists for 5-hydroxytryptamine. Quart. J. Exp. Physiol. 40, 49-74.

Gill, E.W., and H.P. Rang (1966). An alkylating derivative of benzilycholine with specific and long-lasting parasympatholytic activity. Mol. Pharmacol. 2, 284-297.

Giller, E., Jr., and J.H. Schwartz (1971a). Choline acetyltransferase in identified neurons of abdominal ganglion of Aplysia californica. J. Neurophysiol. 34, 93-107.

Giller, E., Jr., and J.H. Schwartz (1971b). Acetylcholinesterase in identified neurons of abdominal ganglion of Aplysia californica. J. Neurophysiol. 34, 108-115.

Ginsborg, B.L. (1967). Ion movements in junctional transmission. Pharmacol. Revs. 19, 289-316.

Goldsmith, T.H. (1963). Rates of action of bath-applied drugs at the neuromuscular junction of the frog. J. Physiol. 165, 368-386.

Goodman, L.S., and A. Gilman (eds) (1965). The pharmacological basis of therapeutics. Macmillan, New York. Chapters 21-28, and passim.

Grundfest, H., and M.V.L. Bennett (1961). Studies on the morphology and electrophysiology of electric organs. I. Electrophysiology of marine electric fishes. In: Biolectrogenesis. C. Chagas and A. Paes de Carvalho, eds. Elsevier, Amsterdam, 57-101.

Hamaguchi, K., K. Ikedi, and C,Y. Lee (1968). Optical rotatory dispersion and circular dichroism of neurotoxins isolated from the venom of Bungarus multicinctus. J. Biochem. 64, 503-506.

Hill, A.V. (1928). The diffusion of oxygen and lactic acid through tissues. Proc. Roy. Soc. B 104, 39-96.

Hill, A.V., and L. Macpherson (1954). The effect of nitrate, iodide and bromide on the duration of the active state in skeletal muscle. Proc. Roy. Soc. B 143, 81-102.

Hill, D.K. (1964). The space accessible to albumin within the striated muscle of the toad. J. Physiol. 175, 275-295.

Hille, B. (1968). Pharmacological modifications of the sodium channels of nerve. J. Gen. Physiol. 51, 199-219.

Hubbard, J.I., and T. Yokota (1964). Direct evidence for an action of acetylcholine on motor-nerve terminals. Nature 203, 1072-1073.

Hunt, R., and R. de M. Taveau (1906). On the physiological action of certain cholin derivatives and new methods for determining cholin. Brit. Med. J. 2, 1788-1791.

Ing, H.R., and W.M. Wright (1933). Further studies on the pharmacological properties of onium salts. Proc. Roy. Soc. B 114, 48-63.

Jacobs, N.H. (1967). Diffusion Processes. Springer-Verlag, New York.

Jenkinson, D.H. (1960). The antagonism between d-tubocurarine and substances which depolarize the motor endplate. J. Physiol. 152, 309-324.

Jiménez-Porras, J.M. (1968). Pharmacology of peptides and proteins in snake venoms. Ann. Rev. Pharmacol. 8, 229-318.

Karlin, A. (1969). Chemical modification of the active site of the acetylcholine receptor. J. Gen. Physiol. 54, 2455-2645.

Karlin, A., and M. Winnik (1968). Reduction and specific alkylation of the receptor for acetylcholine. Proc. Nat. Acad. Sci. U.S.A. 60, 668-674.

Karlin, A., and E. Bartels (1966). Effects of blocking sulfhydryl groups and of reducing disulfide bonds on the acetylcholine-activated permeability system of the electroplax. Biochim. Biophys. Acta 126, 525-535.

Karlin, A., J. Prives, W. Deal, and M. Winnik (1970). Counting acetylcholine receptors in the electroplax. In: Ciba Foundation Symposium on Molecular Properties of Drug Receptors. R. Porter and M. O'Connor, Churchill, London, 246-261.

Karlsson, E., H. Arnberg, and D. Eaker (1971). Isolation of the principal neurotoxins of two Naja naja subspecies. European J. Biochem. (in press).

Karlsson, E., D.L. Eaker, and J. Porath (1966). Purification of a neurotoxin from the venom of Naja nigricollis. Biochim. Biophys. Acta 127, 505-520.

Katz, B., and Miledi, R. (1965a). Propagation of electric activity in motor nerve terminals. Proc. Roy. Soc. B 161, 453-482.

Katz, B., and Miledi, R. (1965b). The measurement of synaptic delay and the time course of acetylcholine release at the neuromuscular junction. Proc. Roy. Soc. B 161, 483-495.

Katz, B., and Miledi, R. (1965c). The effect of calcium on acetylcholine release from motor nerve terminals. Proc. Roy. Soc. B 161, 496-503.

Katz, B., and Miledi, R. (1965d). The effect of temperature on the synaptic delay at the neuromuscular junction. J. Physiol. 181, 656-670.

Katz, B., and Thesleff, S. (1957). A study of the "desensitization" produced by acetylcholine at the motor endplate. J. Physiol. 138, 63-80.

Kehoe, J.S. (1967). Pharmacological characteristics and ionic bases of a two component postsynaptic inhibition. Nature 215, 1503-1505.

Kerkut, G.A., and R.W. Meech (1966). The internal chloride concentration of H and D cells in the snail brain. Comp. Biochem. Physiol. 19, 819-832.

Kerkut, G.A., and R.C. Thomas (1964). The effect of anion injection and changes in the external potassium and chloride concentration on the reversal potentials of the IPSP and acetylcholine. Comp. Biochem. Physiol. 11, 199-213.

Koelle, G.B. (1962). A new concept of the neurohumoral functions of acetylcholine and acetylcholinesterase. J. Pharmacol. (London) 14, 65-90.

Kühne, W. (1887). Neue Untersuchungen über motorischen Nervenendingung. Z. Biol. 23, 1-148.

Ladbrooke, B.D., R.M. Williams, D.M. Chapman (1968). Studies on lecithin-cholesterol-water-interactions by differential scanning calorimetry and X-ray diffraction. Biochim. Biophys. Acta 150, 333-340.

Lambert, D.H., and R.L. Parsons (1970). Influences of polyvalent cations on the activation of muscle endplate receptors. J. Gen. Physiol. 56, 309-321.

Langley, J.N. (1906). On nerve endings and on special excitable substances in cells. Proc. Roy. Soc. B 78, 170-194.

Lee, C.Y. (1970). Elapid neurotoxins and their mode of action. Clinical Toxicology 3, 457-472.

Lee, C.Y., and C.C. Chang (1966). Modes of actions of purified toxins from elapid venoms on neuromuscular transmission. Proc. Int. Symp. Animal Venoms. Sao Paulo, Brazil. Mem. Inst. Butantan 33(2), 555-572.

Lee, C.Y., and L.F. Tseng (1966). Distribution of Bungarus multicinctus venom following envenomation. Toxicon 3, 281-290.

Lee, C.Y., L.F. Tseng, T.H. Chiu (1967). Influences of denervation on localization of neurotoxin from elapid venoms in rat diaphragm. Nature 215, 1177-1178.

Lester, H.A. (1970a). Transmitter release by presynaptic impulses in the squid stellate ganglion. Nature 227, 493-496.

Lester, H.A. (1970b). Postsynaptic action of cobra toxin at the myoneural junction. Nature 227, 727-728.

Lester, H.A. (1970c). Spontaneous miniature synaptic potentials and quantal release of acetylcholine in skate electroplaques. Biol. Bull. 139, 428-429.

Lester, H.A. (1971a). Cobra toxin's action on nicotinic acetylcholine receptors. J. Gen. Physiol. 57, 255.

Lester, H.A. (1971b). Cobra toxin binds to nicotinic acetylcholine receptors. Biophys. Soc. Abst., 130a.

Libet, B. (1970). Generation of slow inhibitory and excitatory postsynaptic potentials. Fed. Proc. 29, 1945-1956.

Ling, G., and R.W. Gerard (1949). The normal membrane potential of frog sartorius fibers. J. Cellular Comp. Physiol. 34, 383-396.

Lo, T.B., Y.H. Chen, and C.Y. Lee (1966). Chemical studies of Formosan cobra (Naja naja atra) venom. Part 1. Chromatographic separation of crude venom on CM-Sephadex and preliminary characterization of its components. J. Chinese Chem. Soc. 13, 25-37.

Loewi, O. (1921). Über humorale Übertragbarkeit der Herznervenwirkung. Pflügers Arch. ges. Physiol. 189, 239-242.

Magazanik, L.G., and F. Vyskočil (1970). Dependence of acetylcholine desensitization on the membrane potential of frog muscle fibre and on the ionic changes in the medium. J. Physiol. 210, 507-518.

Manthey, A.A. (1966). The effect of calcium on the desensitization of membrane receptors at the neuromuscular junction. J. Gen. Physiol. 49, 963-976.

Manthey, A.A. (1970). Further studies of the effect of calcium on the time course of action of carbamylcholine at the neuromuscular junction. J. Gen. Physiol. 56, 407-419.

Martin, A.R. (1955). Further study of the statistical composition of the endplate potential. J. Physiol. 130, 114-122.

Martin, A.R. (1966). Quantal nature of synaptic transmission. Physiol. Revs. 46, 51-65.

Meldrum, B.S. (1965a). The actions of snake venoms on nerve and muscle. The pharmacology of phospholipase A and of polypeptide toxins. Pharmacol. Revs. 17, 393-445.

Meldrum, B.S. (1965b). Actions of whole and fractionated Indian cobra (Naja naja) venom on skeletal muscle. Brit. J. Pharmacol. 25, 197-205.

Miledi, R. (1963). An influence of nerve not mediated by impulses. In: The Effect of Use and Disuse on Neuromuscular Functions. E. Gutmann and P. Hnik, eds. Czechosolvak Academy of Sciences, Prague, 35-41.

Miledi, R., P. Molinoff, L.T. Potter (1971). Isolation of the cholinergic receptor protein of Torpedo electric tissue. Nature 229, 554-557.

Nachmansohn, D. (1970). Proteins in excitable membranes. Science 168, 1059-1066.

Nastuk, W.L. (1953). Membrane potential changes at a single muscle endplate produced by a transitory application of acetylcholine with an electrically controlled microjet. Fed. Proc. 12, 102.

Nastuk, W.L. (1967). Activation and inactivation of muscle post-junctional receptors. Fed. Proc. 26, 1639-1646.

Nastuk, W.L., and A.J. Gissen (1965). Actions of acetylcholine and other quaternary ammonium compounds at the muscle postjunctional membrane. In: Muscle. W.M. Paul, E.E. Daniel, G.M. Kay, and G. Monckton, eds. Pergamon, London, 389-401.

Nastuk, W.L., and A.L. Hodgkin (1950). The electrical activity of single muscle fibres. J. Cellular Comp. Physiol. 35, 39-74.

Nastuk, W.L., and R.L. Parsons (1970). Factors in the inactivation of postjunctional membrane receptors of frog skeletal muscle. J. Gen. Physiol. 56, 218-249.

Nishi, S., and K. Koketsu (1966). Late after discharge of sympathetic postganglionic fibers. Life Sci. 5, 1991-1997.

Paton, W.D.M., and E.J. Zaimis (1952). The methonium compounds. Pharmacol. Revs. 4, 219-253.

Peng, M.T. (1960). Effect of Formosan snake venoms on the depolarizing action of acetylcholine at motor endplate. J. Formosan Med. Assoc. 59, 1073-1082.

Pinsker, H., and E. Kandel (1969). Synaptic activation of an electrogenic sodium pump. Science 163, 931-935.

Porath, J. (1966). Mem. Inst. Butantan, Simp. Internac. 33, 379.

Portela, A., R.J. Perez, J. Vaccari, J.C. Perez, and P. Stewart (1970). Muscle membrane depolarization by acetylcholine, choline and carbamylcholine, near and remote from motor endplates. J. Pharmacol. Exp. Ther. 175, 476-482.

Porter, R., and M. O'Connor (eds) (1970). Ciba Foundation Symposium on Molecular Properties of Drug Receptors. Churchill, London.

Purple, R.L. (1964). The integration of excitatory and inhibitory influences in the eccentric cell in the eye of Limulus. Ph.D. Thesis, The Rockefeller Institute, New York.

Rang, H.P., and J.M. Ritter (1969). A new kind of drug antagonism: evidence that agonists cause a molecular change in acetylcholine receptors. Mol. Pharmacol. 5, 394-411.

Rang, H.P., and J.M. Ritter (1970). Receptor mechanisms at the postjunctional membrane. In: Excitatory Postsynaptic Mechanisms. P. Andersen and J.K.S. Jansen (eds). Universitetsforlaget, Oslo.

Reinert, C., and J.M. Stein (1970). Calorimetric detection of a membrane-lipid phase transition in living cells. Science 168, 1580-1582.

Riker, W.F., Jr., and Michiko Okamoto (1969). Pharmacology of motor nerve terminals. Ann. Rev. Pharmacol. 9, 209-241.

Sasaki, T. (1957a). Chemical studies on the poison of Formosan cobra. II. The terminal amino acid residues of purified poison (neurotoxin). J. Pharm. Soc. Japan 77, 845-847.

Sasaki, T. (1957b). Chemical studies on the poison of Formosan cobra. III. Amino acid composition of purified poison (neurotoxin). J. Pharm. Soc. Japan 77, 848-850.

Sato, S., T. Abe, and N. Tamiya (1970). Binding of iodinated erabutoxin b, a sea snake toxin, to the endplates of the mouse diaphragm. Toxicon 8, 313-314.

Sato, S., H. Yoshida, H. Tamiya (1969). Properties and biosynthesis of a neurotoxic protein of the venom of sea snakes Laticauda laticaudata and Laticauda colubrina. Biochem. J. 115, 85-90.

Silman, I., and A. Karlin (1969). Acetylcholine receptor: covalent attachment of depolarizing groups at the active site. Science 164, 1420-1421

Steinbach, A.B. (1968a). Alteration by Xylocaine (Lydocaine) and its derivatives of the time course of the endplate potential. J. Gen. Physiol. 52, 144-161.

Steinbach, A.B. (1968b). A kinetic model for the action of xylocaine on receptors for acetylcholine. J. Gen. Physiol. 52, 162-180.

Steinbach, A.B. (1971). Direct evidence for temperature insensitivity of processes involved in postsynaptic chemical activation. Biophys. Soc. Abst., 131a.

Stephenson, R.P. (1966). A modification of receptor theory. Br. J. Pharmacol. Chemotherap. 11, 379-393.

Strumwasser, F. (1962). Post-synaptic inhibition and excitation produced by different branches of a single neuron and the common transmitter involved. XXII Internat. Physiol. Congr. 2, 801.

Su, C. (1960). Mode of curare-like action of cobra venom. J. Formosan Med. Assoc. 59, 1083-1091.

Su, C., C.C. Chang, and C.Y. Lee (1967). Pharmacological properties of the neurotoxin from cobra venom. In: Animal Toxins. F.E. Russell and P.R. Saunders, eds. Pergamon, Oxford and New York, 259-267.

Takeuchi, A., and Takeuchi, N. (1959). Active phase of frog's endplate potential. J. Neurophysiol. 22, 395-411.

Takeuchi, A., and Takeuchi, N. (1960). On the permeability of the end-plate membrane during the action of transmitter. J. Physiol. 154, 52-67.

Tamiya, N., and H. Arai (1966). Studies on sea snake venom: crystallization of erabutoxin "a" and "b" from Laticauda semifasciate venom. Biochem. J. 99, 624-630.

Tamiya, N., H. Arai, and S. Sato (1967). Studies on sea snake venoms: crystallization of erabutoxins "a" and "b" from Laticauda semifasciata venom and of lacticotoxin "a" from Laticauda laticaudata venom. In: Aminal Toxins. F.E. Russell and P.R. Saunders, eds. Pergamon, Oxford and New York, 249-258.

Tamiya, N., S. Sato, Y. Endo, A. Seto, H. Yoshida (1970). Structure and action of snake neurotoxins. Toxicon 8, 157.

Tauc, L., and J. Bruner (1963). "Desensitization" of cholinergic receptors by acetylcholine in molluscan central neurons. Nature 198, 33-34.

Tauc, L., and H.M. Gerschenfeld (1962). A cholinergic mechanism of inhibitory synaptic transmission in a molluscan nervous system. J. Neurophysiol. 25, 236-262.

Taylor, D.B., and O.A. Nedergaard (1965). Relation between structure and action of quaternary ammonium neuromuscular blocking agents. Physiol. Rev. 45, 523-554.

Taylor, D.B., J. Steinborn, and T.-C. Lu (1970). Ion-exchange processess at the neuromuscular junction of voluntary muscle. J. Pharm. Exp. Therap. 175, 213-227.

Thesleff, S. (1955). The mode of neuromuscular block produced by acetylcholine, nicotine, decamethonium and succinyl-choline. Acta Physiol. Scand. 34, 218-231.

Thesleff, S. (1960). Effects of motor innervation on the chemical sensitivity of skeletal muscle. Physiol. Rev. 40, 734-752.

Trautwein, W., S.W. Kuffler, and C. Edwards (1956). Changes in membrane characteristics of heart muscle during inhibition. J. Gen. Physiol. 40, 135-145.

Triggle, D.J., J.F. Moran, and E.A. Barnard (eds) (1971). Cholinergic Ligand Interactions. Academic Press, New York.

Tseng, L.F., T.H. Chiu, C.Y. Lee (1968). Absorption and distribution of ^{131}I - labeled cobra venom and its purified toxins. Toxic. Applied Pharmac. 12, 526-535.

van Maanen, E.F. (1950). The antagonism between acetylcholine and the curare alkaloids d-tubocurarine, c-curarine - I, c-toxiferine-II and β-erythroidine in the rectus abdominus of the frog. J. Pharmacol. Exp. Therap. 99, 255-264.

Waser, P.G. (1960). The Cholinergic Receptor. J. Pharm. Pharmacol. 12, 577-594.

Waser, P.G., and U. Lüthi (1957). Autoradiographische Lokalisation von ^{14}C-calebassen-Curarin I und ^{14}C-Decamethonium in der motorischen Endplatte. Arch. Intern. Pharmacodyn. 112, 272-296.

Waud, D.R. (1967). The rate of action of competitive neuromuscular blocking agents. J. Pharmacol. Exp. Therap. 158, 99-114.

Waud, D.R. (1968). Pharmacological receptors. Pharmacol. Revs. 20, 49-88.

Yang, C.C., H.J. Yang, and J.S. Huang (1969). The amino acid sequence of cobrotoxin. Biochim. Biophys. Acta. 188, 65-77.

End