Batrachotoxin: Chemistry and Pharmacology

This novel steroidal alkaloid is a valuable tool for studying ion transport in electrogenic membranes.

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For centuries, skin secretions from a small brightly colored frog have been used by Indians of the Choco rain forest of Colombia to prepare the deadly darts for their blowguns. In 1961, a thorough investigation of the active principles was started (*1*), but their pharmacology was not thoroughly investigated until 1968. In this article we present first the chemical elucidation and partial synthesis, then the broad pharmacological aspects underlying the cardio- and neurotoxicity, of these extremely potent steroidal alkaloids.

Chemistry

Isolation and investigation (*1–4*) of the active principles from the skin of the Colombian poison arrow frog (*Phyllobates aurotaenia*) was beset by numerous difficulties. The frog's habitat is in an almost inaccessible region of western Colombia. The animals are hard to obtain in large numbers (*5*) and do not easily survive shipment. Each adult frog contains only 50 μg of a labile active venom and a corresponding amount of less toxic material. Four major bases were finally separated and isolated: *batrachotoxin*, *homobatrachotoxin*, which was previously incorrectly termed isobatrachotoxin (*3*), *pseudobatrachotoxin*, and *batrachotoxinin A*. Table 1 summarizes their properties.

The fact that these bases were available in only very small amounts precluded the use of the classical techniques of chemical degradation and necessitated the preparation of a suitable crystalline derivative for x-ray analysis. In 1967, the least toxic of the bases, batrachotoxinin A, was converted to a crystalline *O*-*p*-bromobenzoate, from which batrachotoxinin A could be regenerated by basic hydrolysis. Karle and Karle have succeeded in performing x-ray analysis of a single tiny crystal of this derivative (*3, 6*) and established its structure as the 20α-*p*-bromobenzoate of a novel steroidal alkaloid (Fig. 1, a and b).

At this point, detailed spectral analysis of (homo)batrachotoxin and batrachotoxinin A and comparison with model compounds established that batrachotoxin is the 20α ester of the pregnane derivative batrachotoxinin A with 2,4-dimethylpyrrole-3-carboxylic acid (Fig. 1c) and that homobatrachotoxin is the 20α ester of batrachotoxinin A with 2-*ethyl*-4-methylpyrrole-3-carboxylic acid (Fig. 1d). The apparent molecular ion of (homo)batrachotoxin at mass 399 (Table 1) was, therefore, not the true molecular ion. The structure of the very unstable pseudobatrachotoxin (Table 1) is still obscure.

These structures were confirmed by partial synthesis of batrachotoxin by the selective acylation of the allylic 20α-hydroxyl of batrachotoxinin A with the mixed anhydride prepared from 2,4-dimethylpyrrole-3-carboxylic acid and ethyl chloroformate. The synthetic ester alkaloid was identical in all respects with natural batrachotoxin.

A variety of analogs and homologs of batrachotoxin were also synthesized and tested for toxicity (Table 2). The fully substituted 2,4,5-trimethylpyrrole-3-carboxylate was found to be twice as toxic as batrachotoxin. The importance of the intact steroid moiety is demonstrated in the loss of toxicity observed on reduction with sodium borohydride of the 3α,9α-hemiketal linkage in batrachotoxin. The resulting 3β,3α-dihydroxy compound, dihydrobatrachotoxin, has only 1/100 of the toxicity of batrachotoxin.

The unusual structural features of this molecule are the 3α,9α-hemiketal linkage, reminiscent of the 4α,9α-hemiketal of the veratrum alkaloids, the seven-membered 14β,18β-heterocyclic ring, the Δ^{16} unsaturation (*7*), and the 20α-(2,4-dialkylpyrrole-3-carboxylate) (*8*) moiety; these features raise many interesting biogenetic and phylogenetic questions. Batrachotoxin-like compounds have thus far been detected only in dendrobatid frogs of the genus *Phyllobates* and may prove to be unique for this group. The related frogs of the genus *Dendrobates* contain not batrachotoxin but novel decahydro- and octahydroquinoline alkaloids (*9*). The third genus of dendrobatid frogs, *Colostethus*, are virtually devoid of alkaloids.

Pharmacology

Batrachotoxin blocks neuromuscular transmission irreversibly and evokes a muscle contracture in an isolated nerve-muscle preparation (*1*). The mechanism of this neuromuscular block has now been investigated by pharmacological, biochemical, and ultrastructural techniques. Batrachotoxin has emerged from these studies as an important tool for the study of ion transport in nerve, synapse, and muscle.

After intravenous administration (*10, 11*) batrachotoxin causes various cardiac arrhythmias, such as premature ventricular systoles and ventricular tachycardia. These abnormalities terminate in ventricular fibrillation and death at intravenous doses of > 0.1 μg/kg (*12*). After subcutaneous injection most of the symptomatology, such as impairment of coordination and equilibrium, prostration, dyspnea, and clonic convulsions, is compatible with cardiotoxicity and with the resultant hypotension and histotoxic anoxia.

Batrachotoxin inhibits neither Na^+, K^+–activated adenosine triphosphatase from brain or muscle (*13*) nor acetylcholinesterase (*14*). Hydrolysis of cyclic adenosine monophosphate (AMP) by brain cyclic 3′,5′-nucleotide phosphodiesterase is competitively inhibited by relatively high concentrations (*15*).

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Block of Neuromuscular Transmission

Batrachotoxin first blocks the twitch response of muscle fibers to (indirect) stimulation of the nerve (*16, 17*), while the response to direct stimulation of muscle gradually declines during a slowly developing contracture (Fig. 2). The final block is not reversible even after the preparation is washed for a period of 3 hours. At lower temperatures, the effects of batrachotoxin are delayed and diminished. The time course of events is accelerated by higher frequency of stimulation.

The final block of transmission is of presynaptic origin, since microiontophoretic application of acetylcholine at end plate regions of muscles treated with batrachotoxin shows normal sensitivity to this substance (*17*). Even after complete depolarization of the muscle fiber with batrachotoxin, electrical repolarization of the membrane and addition of acetylcholine demonstrate an unaltered sensitivity of the end plate.

Release of transmitter substance from the presynaptic terminal involves (i) an increase in membrane permeability to Na^+ resulting in depolarization; (ii) a Ca^{2+}-dependent coupling of depolarization to transmitter release. The effects of alterations in ion concentrations (Na^+, K^+, Ca^{2+}) on neuromuscular block evoked by batrachotoxin were, therefore, examined. The neuromuscular blockade due to $2 \times 10^{-8}M$ batrachotoxin is delayed about 15 minutes by reduction of Na^+ concentration to 40 m*M*. The absence of K^+ has no effect. Onset of neuromuscular blockade is delayed when the concentration of Ca^{2+} is increased to 15 m*M* and the contracture does not appear. The effects of batrachotoxin appear to be dependent on Na^+ and not on Ca^{2+} (*16, 17*).

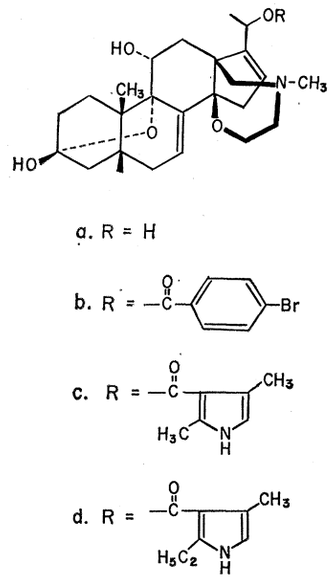

Fig. 1. Structures of (a) batrachotoxinin A; (b) the 20α-*p*-bromobenzoate of batrachotoxinin A; (c) batrachotoxin; (d) homobatrachotoxin.

Depolarization of Muscle Membrane and Muscle Contracture

Batrachotoxin causes two phases of muscle contracture (Fig. 3) (*16*). The first transient contracture is coincident with a decrease in resting membrane potential of the muscle fiber. Depolarization and the first contracture are not transmitter-induced, since *d*-tubocurarine in concentrations sufficient to block indirectly elicited muscle contraction is without effect. When the muscle membrane is first depolarized by 100 m*M* K^+, batrachotoxin does not cause the first contracture. Thus, the first contracture seems clearly due to membrane depolarization. The first contracture is greatly reduced by lowering concentrations of Na^+ to 5 m*M* while the second contracture is unchanged. During a period of 3 hours, $1.6 \times 10^{-7}M$ batrachotoxin fails to produce any contracture in the presence of 15 m*M* Ca^{2+}.

Caffeine and high K^+ elicit muscle contracture by different mechanisms: caffeine by releasing bound Ca^{2+} from the sarcotubular system (*18*), and K^+ by causing membrane depolarization. Neither K^+ nor caffeine elicit contracture after prior treatment of the preparation with $4 \times 10^{-8}M$ batrachotoxin for 2 hours. Batrachotoxin apparently prevents the action of K^+ by depolarizing the muscle membrane and prevents the action of caffeine by disrupting the sarcotubular systems and releasing its stores of Ca^{2+} (*17*). This released Ca^{2+} is probably responsible for the prolonged phase of muscle contracture seen with batrachotoxin.

Tetrodotoxin ($6 \times 10^{-6}M$) completely blocks the effects of $1.6 \times 10^{-7}M$ batrachotoxin on resting membrane potential and on muscle tension. If the muscle is, however, washed to remove the toxins, the membrane potential falls immediately, and the first and second phases of contracture appear quickly. The reversible antagonism by tetrodotoxin, or with low levels of Na^+, lead us to the conclusion that batrachotoxin causes depolarization by an irreversible increase in membrane permeability to Na^+.

Table 1. Properties of the cardiotoxic alkaloids isolated from the Colombian poison arrow frog, *Phyllobates aurotaenia*. Isolation consisted of concentrating methanolic skin extracts at reduced pressure, followed by partition between chloroform and water. The basic principles were extracted from the chloroform layer into 0.1*N* HCl. After basification with 1*N* NH_4OH, they were reextracted into chloroform. Subsequent purification was carried out by preparative thin-layer or column chromatography on silica gel (*4*). DMAB, dimethylaminobenzaldehyde; DMAC, dimethylaminocinnamaldehyde. The numbers in parentheses indicate molecular weight.

Alkaloid	LD_{50} in mice (μg/kg)	Amount/ frog (μg)	Pure compound from 5000 frogs (mg)	Mass spectrum		Ultraviolet spectrum		Infrared spectrum (cm^{-1})	Ehrlich reaction		R_f*
				Apparent molecular ion	Mass	*A* (nm)	ε		DMAB	DMAC	
Batrachotoxin (538)	2	~ 20	11	$C_{24}H_{33}NO_4$	399	234 267	9200 5100	1690	Red	Blue	.52
Homobatrachotoxin (552)	3	~ 10	16	$C_{25}H_{33}NO_4$	399	234 264	9800 5100	1690	Red	Blue	.57
Pseudobatrachotoxin		~ 20	1†	$C_{24}H_{33}NO_4$	399	End absorption			None	None	.54
Batrachotoxinin A (417)	1000	~ 30	47	$C_{24}H_{35}NO_5$	417	End absorption		No carbonyl	None	None	.35

* Silica-gel thin-layer chromatoplates, chloroform and methanol, 7 : 1, detection with sulfuric acid. † Most of pseudobatrachotoxin is converted to batrachotoxinin A during purification.

Muscle Ultrastructure

Batrachotoxin causes swelling and disruption of the terminal cisternae and of the longitudinal part of the sarcoplasmic reticulum (*16*). Tetrodotoxin completely blocks these ultrastructural changes, but after the preparation is washed for 30 minutes, the changes in ultrastructure typical of batrachotoxin treatment are again observable.

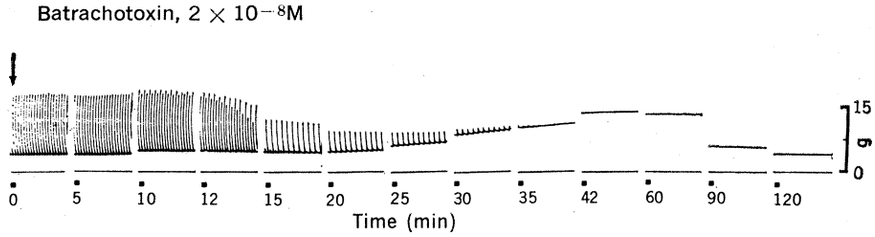

Fig. 2. Blocked indirect and direct muscle twitch of neuromuscular transmission and development of sustained contracture produced by batrachotoxin in the rat phrenic nerve–diaphragm preparation at 37°C. Muscle twitch was recorded isometrically. Direct and indirect stimuli were alternating, each applied at a frequency of 6 per minute.

Transmitter Release

In neuromuscular preparations, batrachotoxin causes a large transient increase in spontaneous release of acetylcholine (*17*) (Fig. 4). The total number of transmitter quanta released by batrachotoxin is far less than the total releasable store of acetylcholine. Additional depolarization with 40 m*M* KCl causes no further transmitter release (Fig. 4). The block of spontaneous transmitter release is not reversed even after prolonged washing. These effects of batrachotoxin probably correlate with levels of depolarization of the presynaptic terminal; the initial depolarization causes transmitter release, and further depolarization blocks release completely.

The end-plate potential amplitude evoked by indirect stimulation in a partially curarized preparation increases greatly in the presence of batrachotoxin during the period corresponding to increased transmitter release. After an additional 20 minutes no end-plate potential can be elicited. Nerve action potentials can still be evoked at this time, and the end plate is still sensitive to acetylcholine. Thus the block in neuromuscular transmission is clearly due to prevention of transmitter release.

Rat diaphragm muscle fibers, whose membrane potential is electrically maintained at −75 mv, respond to direct stimulation with action potentials that gradually disappear only after incubation for 35 to 40 minutes with $10^{-8}M$ batrachotoxin. In rat extensor muscle fibers, $2 \times 10^{-8}M$ batrachotoxin does not affect the action potential–generating mechanism during a 70-minute period. At a concentration ($1.3 \times 10^{-7}M$) which increases spontaneous transmitter release after 60 to 65 minutes at 22°C in frog sartorius muscle, batrachotoxin causes only an increase in the repolarization phases of

Table 2. Effect of the ester moiety on the toxicity of batrachotoxinin A esters.

20α Ester moiety	LD_{50} in mice subcutaneous (μg/kg)*
None (batrachotoxinin A)	1000
2,4-Dimethylpyrrole-3-carboxylate (batrachotoxin)	2
2-Ethyl-4-methylpyrrole-3-carboxylate (homobatrachotoxin)	3
2,5-Dimethylpyrrole-3-carboxylate	2.5
4,5-Dimethylpyrrole-3-carboxylate	260
2,4,5-Trimethylpyrrole-3-carboxylate	1
2,4-Dimethyl-5-ethylpyrrole-3-carboxylate	8
2,4-Dimethyl-5-acetylpyrrole-3-carboxylate	250
1,2,4,5-Tetramethylpyrrole-3-carboxylate	>1000
Pyrrole-2-carboxylate	>1000

* For comparison, the median lethal dose LD_{50} of tetrodotoxin is 8 μg/kg; of curare it is 500 μg/kg; of strychnine it is 500 μg/kg; and of sodium cyanide it is 10,000 μg/kg.

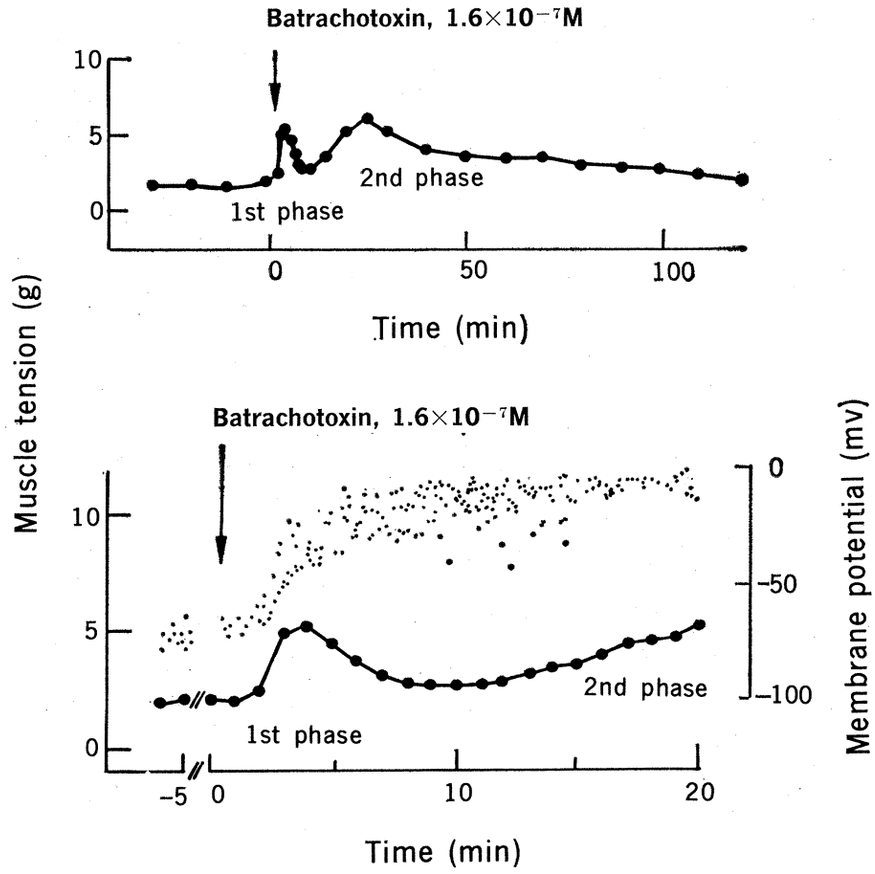

Fig. 3. Two phases of contracture of the nonstimulated diaphragm muscle strip produced by batrachotoxin and correlation of the first phase of muscle contracture with depolarization of the muscle membrane. The resting membrane potential is presented on the second extended scale as a scattergram of measurements in individual surface fibers (*16*).

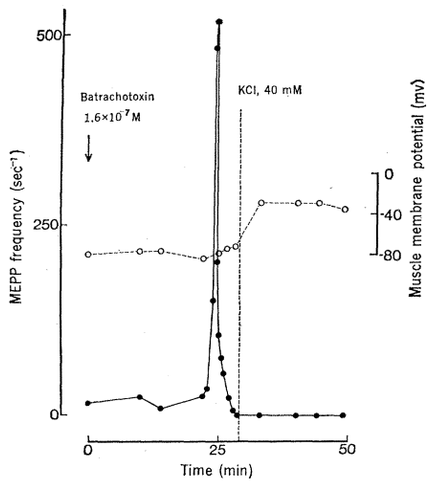

Fig. 4. Increase and block of spontaneous transmitter release [miniature end-plate potentials (MEPP) frequency, ●——●] produced by batrachotoxin in the rat extensor muscle preparation and subsequent effect of 40 m*M* K^+ on spontaneous transmitter release and resting muscle membrane potential (○——○). The transient increase in spontaneous transmitter release was accompanied by an increase in MEPP amplitudes indicative of multiquantal release, and by a slight reduction in muscle membrane potential. After transmitter release was blocked, the muscle was bathed in Ringer solution containing 40 m*M* K^+. Although the resting membrane potential then declined, transmitter release did not occur.

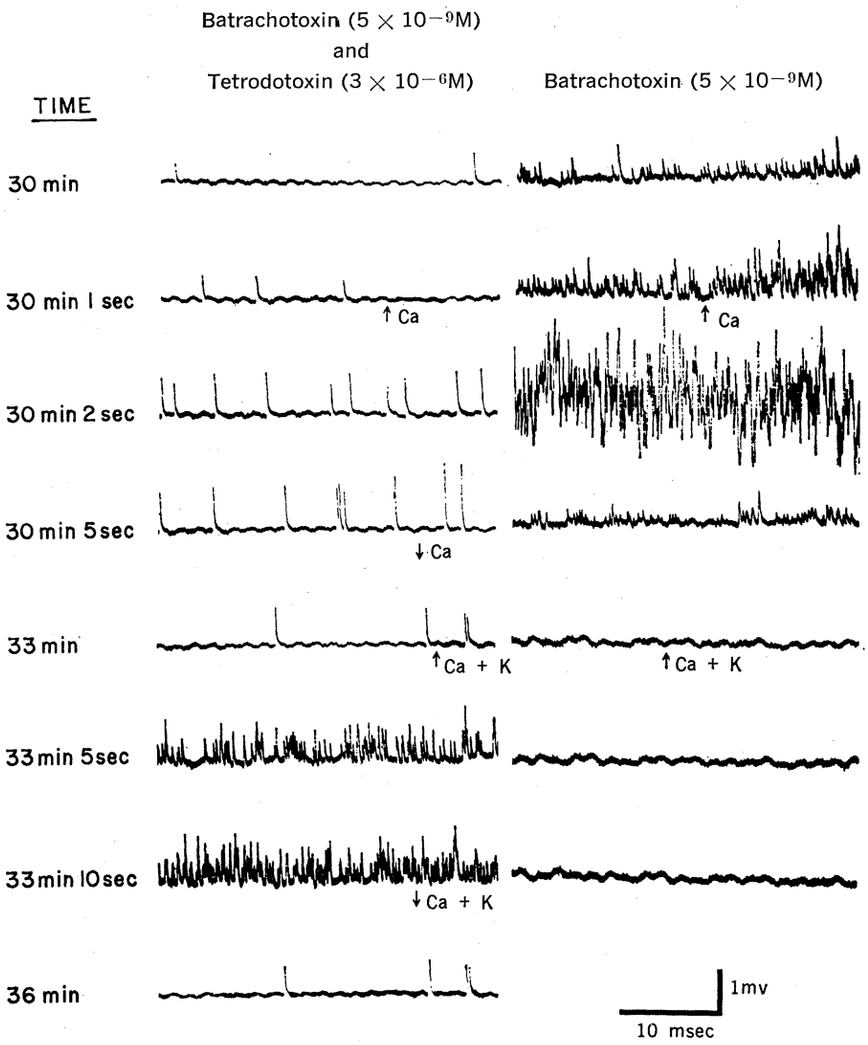

Fig. 5. Effect of microiontophoretically applied Ca^{2+} and K^+ on spontaneous transmitter release in presence of batrachotoxin or of batrachotoxin and tetrodotoxin in end plate regions of rat diaphragm muscles which had been depleted of Ca^{2+} by exposure for 1 hour to a Ca^{2+}-free Ringer solution containing 5 m*M* EGTA (*17*). Tetrodotoxin was present for 30 minutes before batrachotoxin was added. The upper trace was recorded 30 minutes after exposure to batrachotoxin. Initiation of microiontophoresis of ions is indicated by an upward arrow, and cessation is indicated by a downward arrow.

the action potential after 60 minutes. The action potential–generating mechanism thus appears relatively insensitive to the effects of batrachotoxin.

Batrachotoxin ($1.6 \times 10^{-7}M$) does not cause liberation of acetylcholine or depolarization in a Na^+-deficient preparation during 30 minutes. Replacement of the medium with standard Ringer solution now results in rapid depolarization of muscle membrane without concomitant transmitter release. Subsequent washing and incubation with Ringer solution containing 5 m*M* Na^+ leads to repolarization of the membrane.

The onset of enhanced spontaneous transmitter release evoked by $1.6 \times 10^{-7}M$ batrachotoxin at 4.5 minutes is delayed by the presence of 15 m*M* Ca^{2+} to 15 minutes. Depolarization of the muscle fiber is also delayed. During a 3-hour period, batrachotoxin at lower concentrations ($4 \times 10^{-8}M$) has no effect in the presence of 15 m*M* Ca^{2+}. When this preparation is returned to normal Ringer solution, a rapid depolarization and remarkable transient increase in transmitter release occurs.

The transient increase in spontaneous transmitter release and the reduction of resting membrane potential induced by batrachotoxin occurs in Ca^{2+}-free Ringer solution in less than one-half the normal time. However, in Ca^{2+}-free Ringer solution containing 5 m*M* ethylenebis(oxyethylenenitrilo)-tetraacetate (EGTA) batrachotoxin causes only a moderate increase in transmitter release in the course of 30 minutes (Fig. 5). Microiontophoretic application of Ca^{2+} at the end-plate region results in a marked, transient increase in transmitter release. These results with batrachotoxin parallel the well-known requirement for Ca^{2+} ions in transmitter release as evoked by nerve stimulation, or by K^+. After block of transmitter release, depolarization with 40 m*M* K^+ causes no further release.

Tetrodotoxin does not interfere with liberation of acetylcholine or with local transient depolarization of the postsynaptic membrane caused by acetylcholine (*19*). Incubation of the nerve-muscle preparation with tetrodotoxin ($3 \times 10^{-6}M$) blocks action potentials within 3 to 5 minutes. After washing, action potentials do not begin to return to control levels until after 60 minutes. Tetrodotoxin antagonizes the effects of batrachotoxin in nerve-

muscle preparations. Accordingly, spontaneous transmitter release does not increase in the presence of both $1.6 \times 10^{-7}M$ batrachotoxin and $3 \times 10^{-6}M$ tetrodotoxin, and the muscle fiber is depolarized only slightly after 60 minutes. If the preparation is now washed, transmitter release increases transiently after 11 minutes, and this increase is followed by blockade. After 15 minutes, the fiber is completely depolarized. The restoration of action potential (60 minutes) generation and of batrachotoxin-evoked depolarization (15 minutes), after the removal of tetrodotoxin, requires further investigation. It is possible that different operational channels for permeability to Na^+ are involved. Resting membrane potential and spontaneous transmitter release can be restored in preparations depolarized with batrachotoxin by subsequent incubation with tetrodotoxin (Fig. 6). These same preparations are again depolarized if the tetrodotoxin-containing medium is replaced with Ringer solution. Depolarization is delayed by 15 mM Ca^{2+} (Fig. 6).

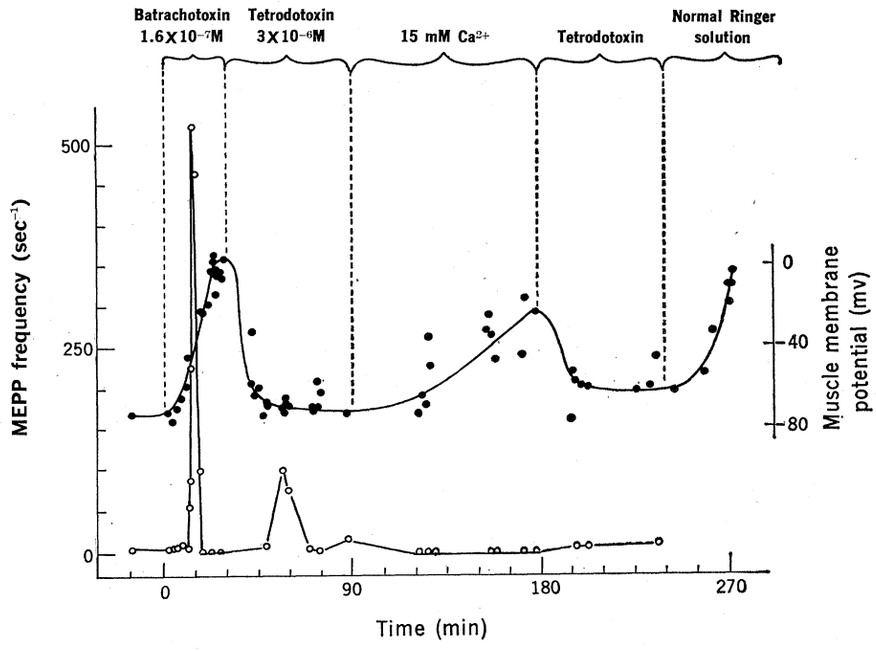

Fig. 6. Time course for the effect of batrachotoxin on spontaneous transmitter release (MEPP frequency, ○——○) and resting membrane potential (●——●) and subsequent effect of tetrodotoxin in the rat diaphragm muscle (*17*). Half circles indicate no transmitter release.

In the Ca^{2+}-free medium containing EGTA, tetrodotoxin blocks the batrachotoxin-evoked increase in spontaneous transmitter release even after application of Ca^{2+} by microiontophoresis (Fig. 5). The simultaneous application of both Ca^{2+} and K^+ in this preparation, however, evokes a great increase in transmitter release. Tetrodotoxin, therefore, blocks depolarization evoked by batrachotoxin, but not that evoked by K^+.

Effect on Ultrastructure of Motor End Plate

Batrachotoxin ($1.6 \times 10^{-7}M$) causes swelling of nerve terminals and deformation and darkening of synaptic vesicles (*17*). Neural mitochondria are enlarged and contain disorganized and vesiculated cristae. The synaptic cleft and pre- and postsynaptic membranes are unaffected.

Nerve Axons

Batrachotoxin causes gradual but complete depolarization of squid giant axons (*20*) at 15° to 23°C (Fig. 7). Depolarization occurs more rapidly after internal application of batrachotoxin in contrast to ouabain, which is inactive inside the axon (*21*). Resting membrane potential is not restored after extensive washing of the preparation.

The depolarizing effect of batrachotoxin is blocked, and axons that have been previously depolarized with batrachotoxin become hyperpolarized in the presence of 1 mM Na^+. Tetrodotoxin prevents the effects of batrachotoxin and causes rapid repolarization of axonal membranes that have been depolarized with batrachotoxin (Fig. 7). Only externally applied tetrodotoxin is effective.

During the course of depolarization, the action potential evoked by electrical stimulation gradually decreases in amplitude and is eventually blocked. Repetitive stimulation accelerates depolarization in the presence of batrachotoxin. If the membrane is now repolarized electrically, action potentials are restored, but the terminal portion of the repolarization phase of the action potential is greatly prolonged, often as long as 1 minute, in contrast to less than 1 second in the normal squid axon.

Batrachotoxin ($2 \times 10^{-7}M$) does not affect the action potential in frog sciatic nerve at 22°C within 90 minutes. After 2 hours at 22°C, $4 \times 10^{-5}M$ batrachotoxin fails to affect the nerve action potential in the toad sciatic-sartorius preparation (*1*). The response of the sartorius muscle to direct stimulation is also virtually unchanged, but neuromuscular transmission is blocked. The results suggest that in nerve, as in muscle, the action potential–generating mechanism is relatively insensitive to batrachotoxin.

Skin Conductance

Batrachotoxin ($2 \times 10^{-6}M$) does not increase the short-circuit current in isolated ventral skin of *Rana pipiens* (*22*). Since short-circuit current under these circumstances corresponds to net sodium ion transferred through the skin from the outer to inner surface, this observation confirms the fact that batrachotoxin does not inhibit Na^+, K^+–activated adenosine triphosphatase.

Superior Cervical Ganglion and Heart Purkinje Fibers

The effect of batrachotoxin on the superior cervical ganglion and on cardiac function has been measured in situ (*10*). Batrachotoxin (0.3 μg, by intraarterial injection) reduces the amplitude of the initial ganglionic spike potential, while apparently increasing

the amplitude of the late ganglionic spike potential (Fig. 8A). The prolonged positive after-potential is markedly reduced by batrachotoxin (Fig. 8A). The results suggest that the activation of fast-conducting preganglionic fibers is depressed by batrachotoxin to a much greater extent than is the activation of slow conducting C fibers. In the presence of batrachotoxin, ganglionic blockade develops rapidly during tetanic stimulation (10 per second), along with a progressive depolarization of the ganglion. In this dose batrachotoxin does not cause any remarkable effect on heart rate or blood pressure. Higher doses (1 to 3 μg) cause ventricular arrhythmias and a complete block of atrioventricular conduction.

Dog heart Purkinje fibers are quickly and irreversibly depolarized by batrachotoxin (*23*). The action potential is altered, especially the repolarization phase (Fig. 8B). The fibers continue to beat spontaneously and respond to stimuli until the resting membrane potential has decreased to values of < -20 mv. Even then, action potentials can be evoked after the membrane is repolarized electrically.

Purkinje fibers depolarized with batrachotoxin (4×10^{-9} g/ml) repolarize in sucrose solution containing 1 mM Na^+. After being returned to normal Tyrode solution these fibers rapidly depolarize within 20 seconds. Higher concentrations of Ca^{2+} delay the onset and extent of depolarization educed by batrachotoxin.

Tetrodotoxin ($5 \times 10^{-6}M$) does not block the action potential in Purkinje fibers. Tetrodotoxin, however, prevents the depolarizing effect of $2 \times 10^{-9}M$ batrachotoxin, again suggesting that different operational Na^+ channels are involved in action potential generation and in batrachotoxin-evoked depolarization. Replacement of the medium, containing batrachotoxin and tetrodotoxin, with standard Tyrode solution leads to a rapid depolarization of the fiber. Fibers will again repolarize in tetrodotoxin-containing medium.

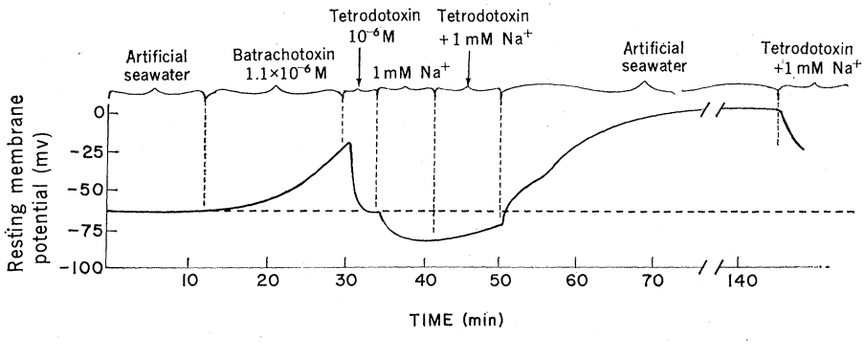

Fig. 7. Effect of batrachotoxin, tetrodotoxin, and low concentrations of Na^+ on the resting membrane potential of an intact squid axon (*20*).

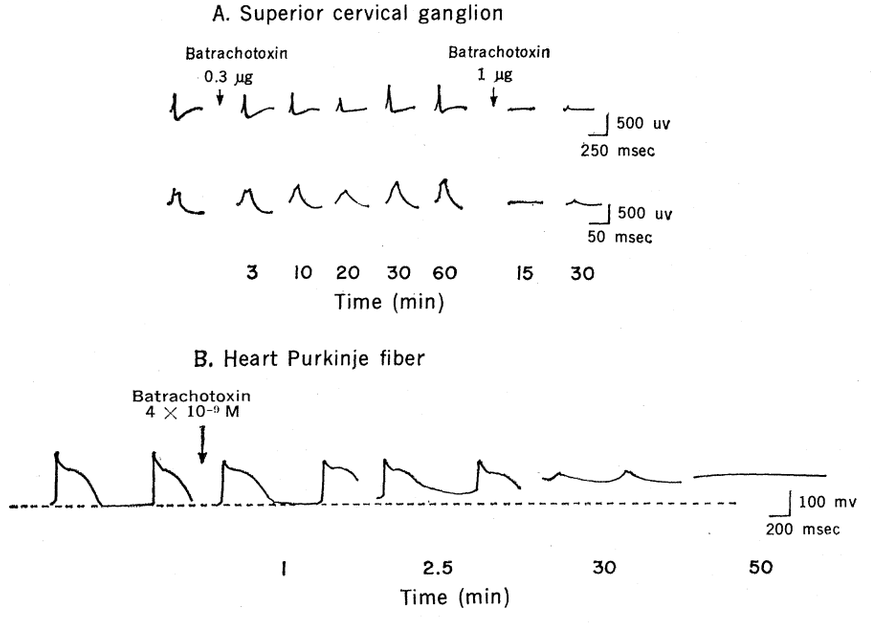

Fig. 8. Effect of batrachotoxin on superior cervical ganglion and heart Purkinje fiber. (A) Effect on ganglionic potentials in the intact rabbit as evoked by supramaximal (10-volt) stimulation of the cervical sympathetic trunk (*10*). Two different sweep rates are depicted. (B) Effect on action potential in the heart Purkinje fiber from dog (*23*). Preparation driven by external stimulus at rate of 95 pulse/min.

Brain

The effect of batrachotoxin on membrane potentials or nerve transmission in the central nervous system has not been measured. However, indirect evidence suggests that in brain slices of cerebral cortex, batrachotoxin causes depolarization of membranes (*15, 24, 25*). This evidence is based on the observation that the accumulation of cyclic adenosine monophosphate (AMP) in brain slices is greatly augmented under conditions which depolarize membranes, that is, electrical stimulation (*26*), or incubation with batrachotoxin, ouabain, veratridine, or increased concentrations of K^+. The mechanism linking membrane depolarization to enhanced accumulation of cyclic AMP is at present unknown, but has been postulated to be mediated by enhanced extracellular accumulation of adenosine under depolarizing conditions (*27*).

The effects of depolarizing agents on accumulation of cyclic AMP have been studied in greatest detail in slices of guinea pig cerebral cortex that had been first labeled with [^{14}C]-adenine (*28*). The percent conversion of a small pool of radioactive adenine nucleotides to cyclic [^{14}C]AMP provides a simple measure of the effect of stimulatory factors. The morphological location of this pool is probably synaptic, since this is where the enzyme adenyl cyclase is found (*29*). Batrachotoxin, in lower concentrations than other depolarizing agents, causes a maximal conversion of 20 percent of the already labeled pool to cyclic [^{14}C]-AMP (Fig. 9). The enhanced accumulation of cyclic [^{14}C]AMP elicited by batrachotoxin and other depolarizing agents requires Ca^{2+}. The parameters that affect the accumulation of cyclic AMP elicited by batrachotoxin

and by other depolarizing agents are the same, that is, inhibition by theophylline and synergistic potentiation by biogenic amines.

The accumulation of cyclic AMP elicited by batrachotoxin is completely blocked by tetrodotoxin and partially blocked in medium in which Na^+ concentration has been reduced (*15*). The effect of batrachotoxin is partially blocked by 15 m*M* Ca^{2+} (Fig. 9). If preparations in which the accumulation of cyclic AMP elicited by batrachotoxin has been blocked by the presence of tetrodotoxin are transferred to normal Krebs-Ringer solution, an accumulation of cyclic [^{14}C]AMP occurs with a magnitude and time course similar to that in slices incubated with batrachotoxin in the absence of tetrodotoxin (*15*).

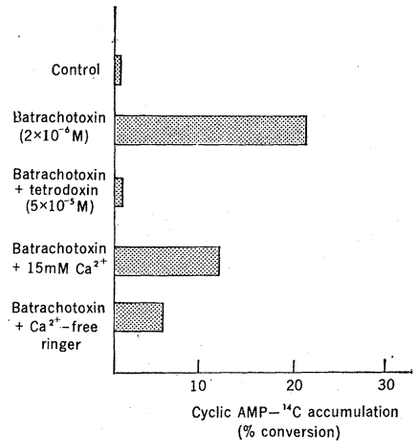

Fig. 9. Effect of batrachotoxin on the accumulation of cyclic [^{14}C]AMP in slices of guinea pig cerebral cortex that had been labeled by incubation with [^{14}C]-adenine (*15*).

Discussion

The effects of batrachotoxin in a variety of systems can be explained either as direct or indirect consequences of the depolarization of electrically excitable membranes. The mechanism of action of batrachotoxin in eliciting membrane depolarization, therefore, assumes prime importance. The hypothesis that batrachotoxin causes a marked dose-dependent and irreversible increase in permeability of membranes to Na^+ is compatible with all of the observations at the present time. Indeed, the magnitude of the depolarization elicited by batrachotoxin in the giant squid axon (*20*) and Purkinje fiber (*23*) can be explained only on the basis of an increase in membrane permeability to Na^+.

The hypothesis is especially attractive in view of the fact that tetrodotoxin is a specific antagonist of batrachotoxin. Tetrodotoxin is known to interfere with generation of action potentials in nerve and muscle by blocking passive diffusion of sodium ions into the cells (*30*). Batrachotoxin is, as expected, less effective in media containing low levels of Na^+. The partial inhibitory effect of Ca^{2+} on the action of batrachotoxin may be explained by its membrane-stabilizing properties, which antagonize permeation of membranes by other ions such as Na^+ (*31*). Such antagonisms might also pertain, if batrachotoxin, like the cardiac glycosides, blocked outflow of Na^+ by inhibition of the Na^+,K^+-activated adenosine triphosphatase (*32*). However, batrachotoxin does not inhibit this enzyme (*13*), nor does it decrease the short-circuit current in membranes as do the cardiac glycosides (*33*).

The time course of events elicited by batrachotoxin in various preparations is increased by concomitant electrical stimulation. This is to be expected, since both batrachotoxin and electrical stimulation tend to increase membrane permeability to Na^+ so that their combined effect should be additive. Alterations in the repolarization phase of action potentials are observed, as would be predicted, for electrogenic membranes with enhanced Na^+ permeability.

The secondary effects of batrachotoxin, such as increase in spontaneous transmitter release and muscle contracture in neuromuscular preparations and cyclic AMP formation in brain slices, appear to result from membrane depolarization. Thus, at a certain critical level of membrane depolarization, spontaneous transmitter release is greatly increased. Cessation of transmitter release may then reflect further depolarization of the presynaptic terminal past this critical level. The first phase of muscle contracture coincides with de-

Table 3. Response of various biological preparations to batrachotoxin and the effect of other parameters.

Response to batrachotoxin	Alteration of response caused by:				
	Electrical stimuli	Low Na^+	High Ca^{2+}	Low Ca^{2+} + EGTA	Tetrodotoxin
		Neuromuscular juncture (rat)			
Block of muscle twitch in response first to indirect stimulation (*A*) and then to direct stimulation (*B*)	Accelerates *A* and *B*	Delays	Delays *A*		
Transient increase in spontaneous transmitter release			Delays	Delays	Prevents
Muscle contracture: first transient phase (*C*); second prolonged phase (*D*)		Antagonizes *C*, little effect on *D*	Delays or prevents	Prevents	Prevents
Depolarization of muscle fiber		Antagonizes	Delays		Prevents
Block of action potential in muscle fiber				Little effect	
Damage to sarcotubular system					Prevents
		Axon (squid)			
Depolarization and resultant block of action potential	Accelerates	Prevents			Prevents
		Intact heart; superior cervical ganglion (rabbit)			
Alteration of action potential (*E*), followed by depolarization (*F*) and block of ganglionic transmission	Accelerates *F*				
		Heart Purkinje fiber (dog)			
Alteration of action potential (*G*), and depolarization (*H*)		Prevents *H*	Delays *H*		Prevents *G*, *H*
		Brain slice (guinea pig)			
Enhanced formation of cyclic AMP		Antagonizes	Antagonizes	Antagonizes	Prevents

polarization of the muscle membrane. The second sustained contracture may be due to disruption of the sarcotubular system with a concomitant release of its stores of Ca^{2+}. This disruption could well be due to osmotic effects of elevated levels of intracellular Na^+. The enhanced formation of cyclic AMP in brain slices in the presence of batrachotoxin is postulated to be due to a depolarization-evoked increase in extracellular levels of adenosine (*25*).

Batrachotoxin irreversibly increases the permeability of membranes to Na^+. Thus, in the muscle fiber and in the squid axon after treatment with batrachotoxin, the membrane can be depolarized or repolarized by manipulation of Na^+ concentration or by addition and removal of tetrodotoxin, a reversible inhibitor.

Although the effect of batrachotoxin on membrane potential and permeability and the antagonistic action of tetrodotoxin, of reduced levels of Na^+ or of elevated levels of Ca^{2+} finds parallels in all preparations investigated (Table 3), the sensitivity of various membranes appears to differ widely. The heart Purkinje cell is extremely sensitive, the neuromuscular preparation is less sensitive, and the squid axon is even less sensitive to batrachotoxin. The sensitivity of preparations to batrachotoxin is markedly reduced (temperature coefficient of about 2.8) at lower temperatures (*16*).

While we still have a poor understanding of the way in which familiar therapeutic agents, such as aspirin and morphine, exert their action, it took less than 3 years to define the basic mechanism of action of batrachotoxin. The selective pharmacological activity of batrachotoxin in irreversibly increasing membrane permeability to Na^+ provides research workers with a valuable tool for the study of electrogenic membranes. Current investigations with synthetic analogs and homologs of batrachotoxin have given promise of developing agents which will have altered activity toward different types of electrogenic membranes. Such agents might eventually find use not only as pharmacological tools but as therapeutic agents.

Summary

Batrachotoxin has been shown to be a pyrrolecarboxylic ester of a novel steroidal base with unique and selective actions on a variety of electrogenic membranes. The effects of batrachotoxin in neuromuscular preparations both pre- and postsynaptically, in nerve axons, in superior cervical ganglion, in heart Purkinje fibers, and in brain slices appear to be due to the selective and irreversible increase in permeability of membranes to sodium ions. The subsequent effects of this increase in Na^+ permeability evoked by batrachotoxin—such as membrane depolarization, enhanced spontaneous transmitter release, muscle contracture, and enhanced formation of cyclic AMP in brain slices—may be blocked reversibly by tetrodotoxin.

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