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Interactions of Gephyrotoxin with the Acetylcholine Receptor-Ionic Channel Complex

I. Blockade of the Ionic Channel

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SUMMARY

The novel tricyclic alkaloid, gephyrotoxin (GyTX), found in the skin secretions of the frog *Dendrobates histrionicus*, potentiates and blocks the indirectly elicited muscle twitch in a concentration-dependent manner. GyTX prolongs the falling phase of the muscle action potential and decreases delayed rectification, supporting the idea that the alkaloid blocks the voltage-sensitive potassium conductance of the electrically excitable membrane. The peak amplitude of the end-plate currents (EPC) and miniature end-plate currents (MEPC) were depressed, but no significant deviaton from linearity relative to control was seen in the current-voltage relationship. The decay time constant of the EPC (τ_{EPC}) was markedly shortened by GyTX, the effect being greater at 10° than at 22°. The relationship between the log of τ_{EPC} and membrane potential disclosed a linear relationship at all concentrations tested, but a progressive loss of voltage sensitivity of τ_{EPC} was seen when GyTX concentrations were increased. Also, the plot of $1/\tau_{EPC}$ against GyTX concentration revealed a linear relationship. The lack of voltage and time dependence suggests that GyTX has little effect on the ACh receptor-ionic channel complex in the closed conformation. Single-channel conductance studied by means of fluctuation analysis did not change after GyTX application, but the channel lifetime decreased by about 40% at clamp potentials of −105 mV and at a toxin concentration of 7.5 μM. Repetitive nerve stimulation led to a pronounced "rundown" in the EPCs which was frequency-dependent. These findings were taken as evidence that GyTX interacts with the acetylcholine receptor complex, causing a blockade of its channel mainly in the open conformation.

INTRODUCTION

GyTX[5] is a novel tricyclic alkaloid, [1*S*,3a*S*,5a*S*,6*S*(*Z*),9a*R*,10*R*]dodecahydro-6-(2-penten-4-ynyl)pyrrolo[1,2-a]quinoline-1-ethanol (Fig. 1A), found in skin secretions of the Colombian poison frog *Dendrobates histrionicus* (1). A relatively large number of alkaloids have been isolated from the skin secretions of frogs of the *Dendrobatidae* family. Among others, HTX and its derivatives (2) appear to have selective effects on nerve and muscle. HTX blocks neuromuscular transmission by interacting with the AChR in both closed and open conformations, increasing receptor affinity for agonists and enhancing desensitization. These effects of HTX led to the suggestion that the alkaloid binds to at least two sites at the AChR (2–6). On the other hand, compounds such as atropine, scopolamine, and quinuclidinyl benzilate, and certain anesthetics, such as piperocaine, appear to interact mainly with the open conformation of the AChR complex and have no effect on the affinity of the receptor for ACh (7–12). Others, like the quaternary local anesthetic meproadifen, have little effect on channel lifetime

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[5] The abbreviations used are: GyTX, gephyrotoxin; HTX, histrionicotoxin; ACh, acetylcholine; AChR, acetylcholine receptor-ionic channel complex; EPP, end-plate potential; EPC, end-plate current; MEPC, miniature end-plate current; τ_{EPC}, decay time constant of EPC; τ_{MEPC}, decay time constant of MEPC; γ, single-channel conductance; τ_l, single channel lifetime.

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or conductance but do increase ACh affinity for the receptor (5, 13).

In this and in the companion paper (14), we describe the effects of GyTX on neuromuscular transmission. It was found that the alkaloid decreases both the peak amplitude of the EPC and the τ_{EPC} of frog sartorius muscle in a concentration-dependent manner. At all concentrations used, GyTX did not cause a marked voltage or time dependence, in contrast to results with HTX or meproadifen (6, 13). GyTX chiefly decreased channel lifetime but had no effect on single-channel conductance, as studied by noise analysis. GyTX also shows a "use-dependent" action; i.e., its action is potentiated by previous channel openings. These and other observations reported in this paper can be taken as evidence for an interaction of GyTX mainly with the open conformation of the AChR complex of the nicotinic synapse. In addition, GyTX also appears to increase the affinity of the nicotinic receptor for ACh as reported for HTX and meproadifen (6, 5). This latter action is discussed in detail in the companion paper (14).

MATERIALS AND METHODS

Experiments were performed on the sciatic nerve-sartorius muscle preparation of the frog *Rana pipiens* at room temperature (20–22°) or as otherwise indicated.

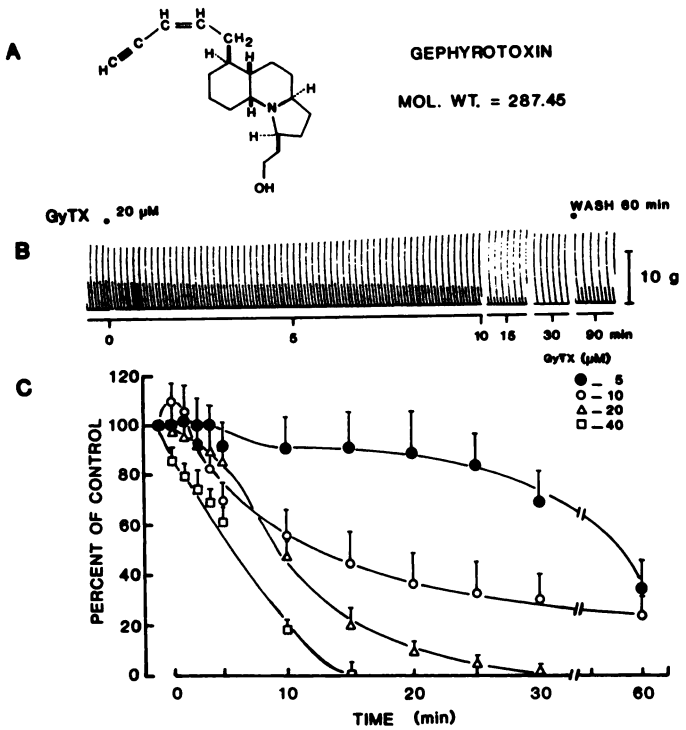

FIG. 1. *Structure of GyTX and effect on evoked twitch of the frog sciatic nerve-sartorius muscle preparation*

A. Structure of GyTX. B. A polygraph record of isometric twitches evoked by supramaximal direct and indirect stimulation. Addition of GyTX (20 μM, *first dot*) blocked the indirect twitch in about 30 min. Washing the preparation for 60 min (*second dot*) with GyTX-free physiological solution causes a partial (60%) recovery of the indirect twitch. Time course of blockade is shown in C for several concentrations of GyTX. Indirect evoked muscle twitches are plotted as percentage of control against time. Note initial facilitation observed at low concentrations of GyTX. Each point represents the mean ± standard error of the mean of observations made in four muscles.

Twitch tension studies were done by stimulating the muscle both indirectly via the sciatic nerve (supramaximal rectangular pulses, duration between 0.05 and 0.2 msec) and directly (supramaximal rectangular pulses, duration between 1 and 2 msec) at a rate of 0.2 Hz. Muscle twitches were recorded on a Grass polygraph. For microelectrode studies, the muscles were pinned down, slightly stretched, to a Sylgard block and kept in a 20-ml bath continuously perfused with physiological solutions. Action potentials were elicited by passing a 30-msec depolarizing pulse via a microelectrode inserted 100 μm away from the recording electrode. The maximal rates of rise and fall of action potentials were measured through an R-C circuit (1 Mohm–100 pF).

Delayed rectification was measured in muscles previously exposed to 0.3 μM tetrodotoxin. Briefly stated, muscle membrane potentials were kept at −90 mV, and hyperpolarizing or depolarizing current pulses (pulse duration lasted for 200 msec) were applied via one microelectrode kept less than 50 μm from a second recording microelectrode. The voltage responses to these pulses were taken as a measure of the inward rectification (15, 16).

EPPs were recorded in preparations previously equilibrated with a physiological solution containing 10 mM Mg^{2+} in order to avoid action potentials and the subsequent contractions (17). The amplitude of the EPPs was corrected for a standard membrane potential (−90 mV) and nonlinear summation following published methods (18, 19). Quantal content of the EPP was calculated from the variance of a train of EPPs with the aid of a PDP 11/40 computer (Digital Equipment Company, Maynard, Mass.).

Evoked EPCs were recorded in muscles previously exposed to 600 mM glycerol for about 60 min and washed with physiological solution for another hour in order to block excitation-contraction coupling (20). The membrane potential of the control glycerol-treated muscle fibers had a mean of −43.0 ± 1.0 mV (30 fibers, 8 muscles) and was not significantly altered after exposure to either concentration of GyTX used. Voltage clamp of end-plate regions was achieved by using a circuit similar to that described elsewhere (21). Two electrodes, one for recording voltage and the other for passing current, were placed in the end-plate region at an interelectrode distance not greater than 30 μm. Microelectrodes were filled with 3 M KCl and had resistances between 1 and 3 Mohm. Series of EPCs were evoked by stimulating the sciatic nerve every 3 sec. Membrane voltage was changed from a holding potential, close to the resting potential, in 10-mV steps in the depolarizing direction and then in the hyperpolarizing direction, covering the range from +50 mV to −150 mV. Where noted, EPCs were elicited also at frequencies of 25, 50, and 100 Hz. When the influence of the voltage-conditioning step length on the relationship between peak EPC amplitude and voltage was examined, we followed the sequence described by Masukawa and Albuquerque (6). After proper amplification, EPCs were digitized at a rate of 10 KHz and analyzed with the aid of the computer. The τ_{EPC} was calculated by linear regression on the logarithms of the digitized points against time.

Spontaneous MEPC and microiontophoretically induced EPC for noise analysis were measured in muscles previously exposed to 0.3 μM tetrodotoxin using conventional techniques (12, 22). MEPCs were recorded on FM tape (Racal Store, Rockville, Md.), played back through a bandpass filter (1–2.5 KHz) (Krohn-Hite 3700 band-pass filter) and captured by a Digital oscilloscope (Gould 0S4000). From the oscilloscope, selected MEPCs were sent to the computer at a sampling rate of 18 KHz for averaging and analysis. Microiontophoretically induced EPCs were stored on tape and also recorded on a Mingograph 81 having a frequency response between d.c. and 700 Hz. Clamp and iontophoretic currents were recorded both on a low-gain d.c. and a high-gain a.c. mode. The high-gain a.c. signal was filtered (1–800 Hz) by a Krohn Hite 3700 band-pass filter. Thirty segments of 0.256 sec each, both of the baseline and of the ACh current fluctuations, were sent to the computer at a sampling rate of 2 KHz. These records were obtained before and after treatment with GyTX in the same cell. The resulting power density spectra were fitted to a Lorentzian curve from which $S(0)$, the zero frequency asymptote, and τ_I, the single channel lifetime,

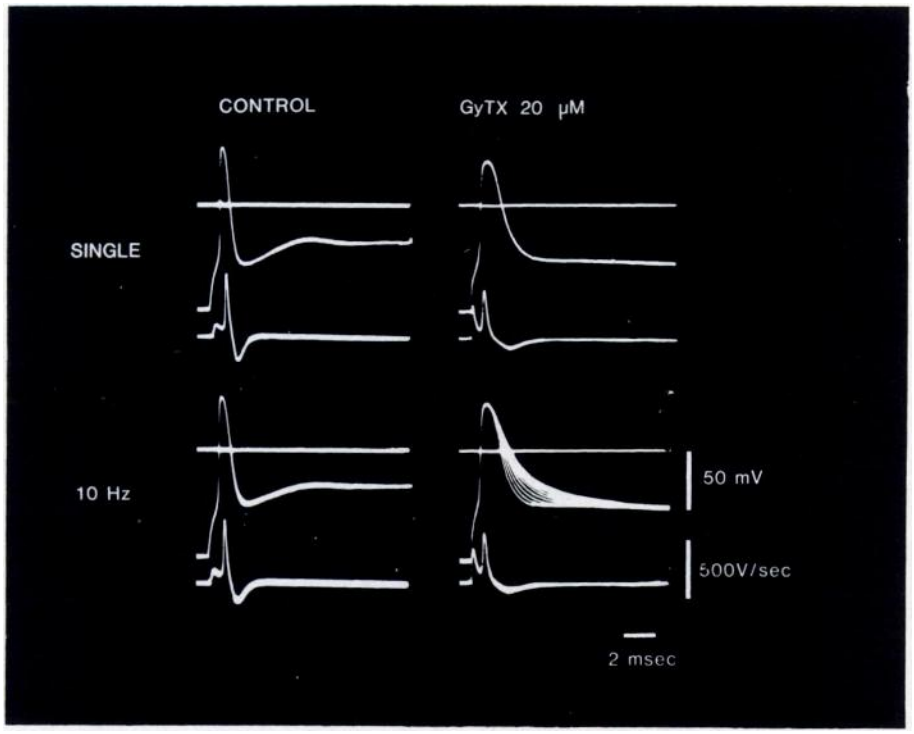

FIG. 2. *Direct evoked action potential recorded at the extrajunctional region of surface fibers of the frog sartorius muscle under control conditions (left) and 30 min after addition of GyTX (20 μM) (right)*

Traces under action potentials are their first derivatives. GyTX clearly induced prolongation of the falling phase while only slightly affecting amplitude and rise time of action potentials. The effect of GyTX was enhanced by increasing the frequency of stimulation to 10 Hz (*lower traces*), thus disclosing a "use-dependent" action. The rate of fall of the tenth action potential induced in a train decreased by 62%. Resting membrane potential was around −95 mV.

were determined. Single-channel conductance was obtained from the power spectrum according to the equation:

$$\gamma = \frac{S(0)}{[4\mu\ (V - V_{eq})\tau_I]}$$

where μ = mean ACh-induced current, V is the clamp potential, V_{eq} is the equilibrium potential of the ACh channel (taken to be −15 mV (23, 24) and $S(0)$ is the zero frequency asymptote of the power spectrum.

Normal frog Ringer's solution used in these experiments had the following composition (millimolar): NaCl, 116; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4, 1.3; NaH_2PO_4, 0.7. After bubbling with 100% O_2, the final pH was between 6.9 and 7.1.

GyTX was kept at 4° as a stock solution (10 mM in 95% ethanol) and diluted prior to use. The lipid-soluble alkaloid was purified according to the method of Daly *et al.* (1). The authors are grateful to Dr. T. Tokuyama (Osaka City University) for additional supplies of GyTX.

Results are expressed as mean ± standard error, and n indicates the number of observations. Where pertinent, Student's t-test was used to compare control and experimental conditions. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of GyTX on muscle twitch and membrane excitability. When applied to the sciatic nerve-sartorius muscle preparation, GyTX blocked the indirect muscle twitch without impairing the directly elicited twitch (Fig. 1B). At low concentrations (5 and 10 μM), the alkaloid induced a transient potentiation in the first 2–3 min after incubation. The extent of blockade of neuromuscular transmission was concentration-dependent. For example, 20 μM GyTX caused complete blockade of twitch in about 30 min, whereas at 40 μM only 15 min were required for complete abolition of the muscle response

TABLE 1

Effect of GyTX on the threshold, amplitude, rate of rise, half-decay time, and rate of fall of the direct elicited action potential of frog sartorius muscle fibers

Measurements were made 30 min after addition of GyTX to the bath and after a 60-min washing of the preparation previously treated with 40 μM GyTX. Values are expressed as means ± standard error of the mean. N is the number of fibers tested in three to five muscles.

Condition	*N*	Threshold	Amplitude	Half-decay time	Rate of rise	Rate of fall
		mV	*mV*	*msec*	*V/sec*	*V/sec*
Control	37	42 ± 0.5	89 ± 1.0	0.64 ± 0.06	544 ± 17	249 ± 15
GyTX, 20 μM	16	42 ± 0.9	90 ± 1.0	1.12 ± 0.08*	468 ± 15*	162 ± 17*
GyTX, 40 μM	33	43 ± 0.6	81 ± 1.0*	0.94 ± 0.08*	429 ± 20*	134 ± 11*
Wash	16	45 ± 1.1	80 ± 1.0*	0.60 ± 0.05	421 ± 19*	224 ± 15

* $p < 0.05$ with respect to control.

(Fig. 1C). Also, at these higher concentrations, no facilitation was seen in the first minutes. Washing the preparation for 60 min with GyTX-free Ringer's solution reversed the blockade by about 50%. Since this effect could have been caused by an action of the alkaloid on the excitability of the muscle membrane, we checked the effect of GyTX on directly evoked action potentials and measured their threshold, rate of rise, amplitude, and rate of fall. As shown in Fig. 2, GyTX (20 μM) prolonged the action potential in a frequency-dependent manner such that at a rate of stimulation of 10 Hz, the half-decay time was prolonged from 1.12 ± 0.08 msec, for the first action potential, to 2.02 ± 0.10 msec (10 cells) for the tenth action potential. Table 1 summarizes these findings. At a concentration of 40 μM, GyTX decreased the amplitude of the action potential by 9%, the rate of rise by 21%, and the rate of fall by 46%. On the other hand, at 20 μM GyTX affected mainly the rate of fall, inducing a 35% decrease after a 30-min incubation. In accordance with this, the half-decay time was increased by 75%. Such effects could be explained either by a slow inactivation of sodium channels or by a blockade of the potassium conductance. Delayed rectification has been taken as an indicator of potassium channel activation, in both squid axon and frog sartorius muscle (15, 16, 25). Figure 3 shows that, after 30 min of incubation with GyTX (20 μM), there was an almost complete block of the inward rectifying current. Higher frequencies of stimulation induced a more rapid blockade. Washing the preparation restored delayed rectification to control levels, contrary to the partial restoration seen with the indirectly elicited twitch. These results indicate that an effect of GyTX on the muscle action potential cannot explain the observed blockade of indirectly elicited twitch. In fact, the effects on potassium conductance provide an explanation for the initial facilitation in the indirectly and directly elicited twitch observed at low doses.

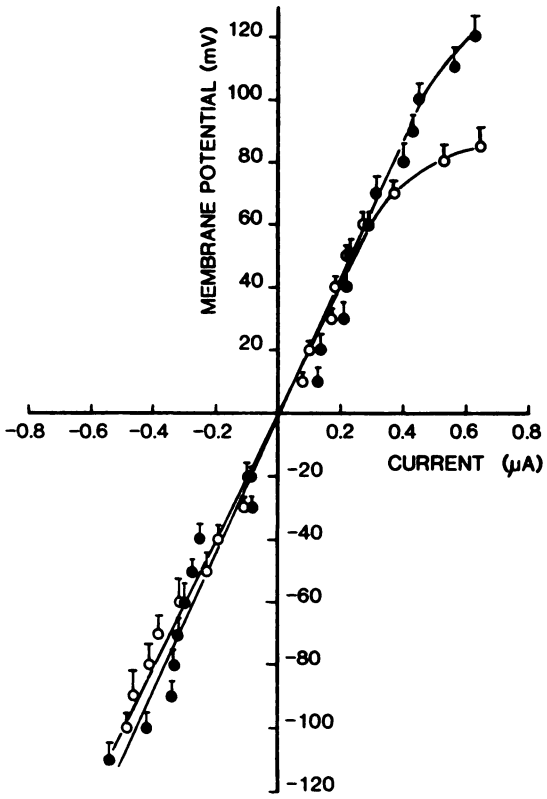

FIG. 3. *Delayed rectification in frog sartorius muscle before (○) and after (●) 30-min exposure to 20 μM GyTX*

Electrotonic potentials were elicited by depolarizing and hyperpolarizing current pulses applied to surface fibers of muscles treated with 0.3 μM tetrodotoxin to block sodium conductance. Each point represents the mean ± standard error of the mean of 25 fiber measurements in at least 5 muscles.

Effect of GyTX on amplitude and time course of spontaneous MEPCs and EPCs. The site of GyTX action appears to be the postjunctional membrane of the endplate, as judged by its marked effect on MEPC and EPC amplitudes and decay time constants (τ_{MEPC} and τ_{EPC}, respectively). Table 2 shows that the amplitude of MEPCs and τ_{MEPC} were reduced by about 25% upon exposure to GyTX (30 μM) for 40 min. The extent of blockade was about the same at both −70 mV and −90 mV clamp potential. On the other hand, the MEPC rise time was not affected by GyTX. The peak EPC amplitude was depressed by GyTX in a concentration-dependent manner (10–50 μM). Each concentration induced a progressive depression in the peak EPC for periods of over 2 hr. Because of this long time required for GyTX action to reach equilibrium, all of the results were obtained after a 30- to 60-min exposure of the preparation to the alkaloid. Apparently this slow equilibration condition is related to the ability of the alkaloid to induce progressive channel blockade coupled with desensitization, as revealed by our recent patch clamp studies, which disclosed that the effect of GyTX on the open-channel conformation occurs immediately after establishment of a gigaohm seal in which GyTX is present inside the patch micropipette together with the agonist (26), whereas the activation and subsequent desensitization appeared more slowly, mainly at low concentrations (1–5 μM). Thus, the initial results on patch clamp using GyTX support previous findings with meproadifen that desensitization (5) and open-channel block are unrelated; i.e., meproadifen, which induces desensitization, affects neither channel conductance nor channel lifetime (13, 27). Table 3 shows the effect of GyTX on the EPC amplitude, rise time, and τ_{EPC} at −90 mV clamp potential, at both 22° and 10°. In keeping with the findings regarding the MEPC, the main effects were on the peak amplitude and τ_{EPC}. The rise time was almost unaffected except at a concentration of 50 μM. At a concentration of 30 μM GyTX, peak EPC amplitude decreased by 50% at both temperatures. τ_{EPC}, on the other hand, was much more affected at 10° (62% decrease at 30 μM) than at 22° (34% decrease at 30 μM). In fact, GyTX (10 μM) was nearly ineffective in decreasing τ_{EPC} at 22° but caused a 30% reduction at 10°. Washing the preparation with normal Ringer's solution for 60 min reversed the effects of GyTX on the peak EPC amplitude at both temperatures. Assuming that channel lifetime is larger at low temperatures, these results suggest that the longer the channel remains open the more efficient is the blocking action of GyTX.

TABLE 2

Effects of GyTX on the amplitude and decay time constant (τ) of spontaneous MEPC in frog sartorius muscle fibers

Measurements were made 30 min after addition of GyTX to the muscle bath or after a 120-min wash of the preparation previously treated with 40 μM GyTX. Values are means ± standard error of the mean. *N* indicates number of MEPCs recorded in 10–15 muscle fibers (5–8 preparations).

Condition	Membrane potential								
	−70 mV			−90 mV			−110 mV		
	N	MEPC amplitude	τ	*N*	MEPC amplitude	τ	*N*	MEPC amplitude	τ
		namp	*msec*		*namp*	*msec*		*namp*	*msec*
Control	307	4.22 ± 0.20	1.17 ± 0.07	339	4.56 ± 0.32	1.32 ± 0.05	244	5.07 ± 0.28	1.56 ± 0.08
GyTX, 5 μM	101	4.02 ± 0.18	1.01 ± 0.15	199	3.82 ± 0.14	1.49 ± 0.07	112	4.48 ± 0.19	1.56 ± 0.20
GyTX, 10 μM	134	4.29 ± 0.28	1.17 ± 0.06	246	3.91 ± 0.29	1.32 ± 0.07	120	4.68 ± 0.48	1.52 ± 0.17
GyTX, 20 μM	194	3.44 ± 0.40	1.01 ± 0.06	221	3.87 ± 0.24	1.10 ± 0.06[a]	225	4.25 ± 0.21	1.19 ± 0.09[a]
GyTX, 30 μM	247	3.18 ± 0.23[a]	0.94 ± 0.05[a]	233	3.48 ± 0.27[a]	0.99 ± 0.09[a]	228	3.58 ± 0.27[a]	1.10 ± 0.11[a]
GyTX, 40 μM	220	3.09 ± 0.19[a]	0.67 ± 0.04[a]	180	3.39 ± 0.31[a]	0.65 ± 0.06[a]	116	3.59 ± 0.73[a]	0.71 ± 0.07[a]
Wash	124	4.69 ± 0.20	0.74 ± 0.02[a]	166	4.64 ± 0.26	0.80 ± 0.04[a]	106	5.87 ± 0.35	0.97 ± 0.05[a]

[a] Significantly different from control ($p < 0.05$).

The I-V relationship of the peak EPC amplitude in control condition and after treating the preparation with several concentrations of GyTX is shown in Fig. 4A. In contrast to the effect of other drugs, such as histrionicotoxins (2, 28), meproadifen (5, 13), tetraethylammonium ions (29), atropine and scopolamine (8), and phencyclidine methiodide (30), GyTX did not seem to have a voltage-dependent effect on the peak amplitude of the EPC. Departure from the linear I-V relationship in the third quadrant was very slight and appeared only at large hyperpolarizing potentials (−160 mV; Fig. 4A). A small departure from a linear I-V relationship has also been reported for control conditions (21, 31). A plot of the logarithm of τ_{EPC} against voltage showed a linear relationship (Fig. 4B), both before and after treatment with GyTX. However, GyTX induced a loss of voltage dependence in τ_{EPC}. Under control conditions, τ_{EPC} changed approximately 2-fold/100 mV, as reported by others (32), whereas after 40 μM GyTX it changed only 2-fold/300 mV. Since the shortening of the decay time constant can occur as the result of open-channel block, two pathways appear to suffice, i.e., a first-order, spontaneous closure, and pseudo-first order reaction with drug (see sequential model under Discussion, and also refs. 8 and 33). This model allows one to test whether or not a linear relationship is present when plots at reciprocal τ versus drug concentration are obtained. Indeed, the *inset* in Fig. 4B shows a plot of $1/\tau_{EPC}$ against GyTX concentration for several clamp potentials. The relationship between them is linear at all voltages, suggesting that GyTX preferentially reacts with the ionic channel in open conformation. The decay of EPCs in the presence of GyTX can still be accurately described by a single exponential function, as shown in Fig. 5 for various clamp potentials.

Experiments were also carried out in order to detect any time-dependent effects of the GyTX. The voltage sequence used to study this phenomenon was similar to that described by Masukawa and Albuquerque (6). When elicited after long-duration (3 sec) conditioning pulses and in the presence of 30 μM GyTX, the EPC peak amplitude I-V relationship disclosed a very slight hysteresis loop. On the other hand, 20-msec conditioning pulses gave only a linear I-V relationship regardless of the

TABLE 3

Effect of GyTX on the peak amplitude, rise time, and decay time constant (τ_{EPC}) of the (EPC) in frog sartorius muscle fibers at −90 mV and two different temperatues

Measurements were taken 30 min after exposure to GyTX or after a 60 min wash the preparation previously treated with 50 μM GyTX. Values are the means ± standard error of the mean. *N* represents the number of cells studied in four to seven muscle preparations.

Condition	Temperature							
	22°				10°			
	N	Peak EPC amplitude	Rise time	τ_{EPC}	*N*	Peak EPC amplitude	Rise time	τ_{EPC}
		μamp	*msec*	*msec*		*μamp*	*msec*	*msec*
Control	21	0.27 ± 0.03	0.94 ± 0.03	1.94 ± 0.06	17	0.27 ± 0.03	2.49 ± 0.12	10.55 ± 1.04
GyTX, 10 μM	11	0.24 ± 0.02	0.96 ± 0.03	1.81 ± 0.08	7	0.21 ± 0.03	2.40 ± 0.23	7.51 ± 1.05[a]
GyTX, 20 μM	11	0.18 ± 0.03	0.81 ± 0.04	1.37 ± 0.08[a]	10	0.20 ± 0.03[a]	2.25 ± 0.25	5.56 ± 0.40[a]
GyTX, 30 μM	18	0.14 ± 0.03[a]	0.80 ± 0.03	1.29 ± 0.07[a]	10	0.16 ± 0.02[a]	2.35 ± 0.09	4.09 ± 0.19[a]
GyTX, 40 μM	15	0.07 ± 0.02[a]	0.83 ± 0.05	1.01 ± 0.06[a]	8	0.06 ± 0.01[a]	2.25 ± 0.13	2.79 ± 0.08[a]
GyTX, 50 μM	8	0.08 ± 0.01[a]	0.65 ± 0.05[a]	0.80 ± 0.09[a]	5	0.07 ± 0.02[a]	2.20 ± 0.20	2.90 ± 0.60[a]
Wash	17	0.24 ± 0.04	0.80 ± 0.03	1.35 ± 0.04[a]	7	0.25 ± 0.07	2.64 ± 0.17	9.83 ± 1.31

[a] Significantly different from control ($p < 0.05$).

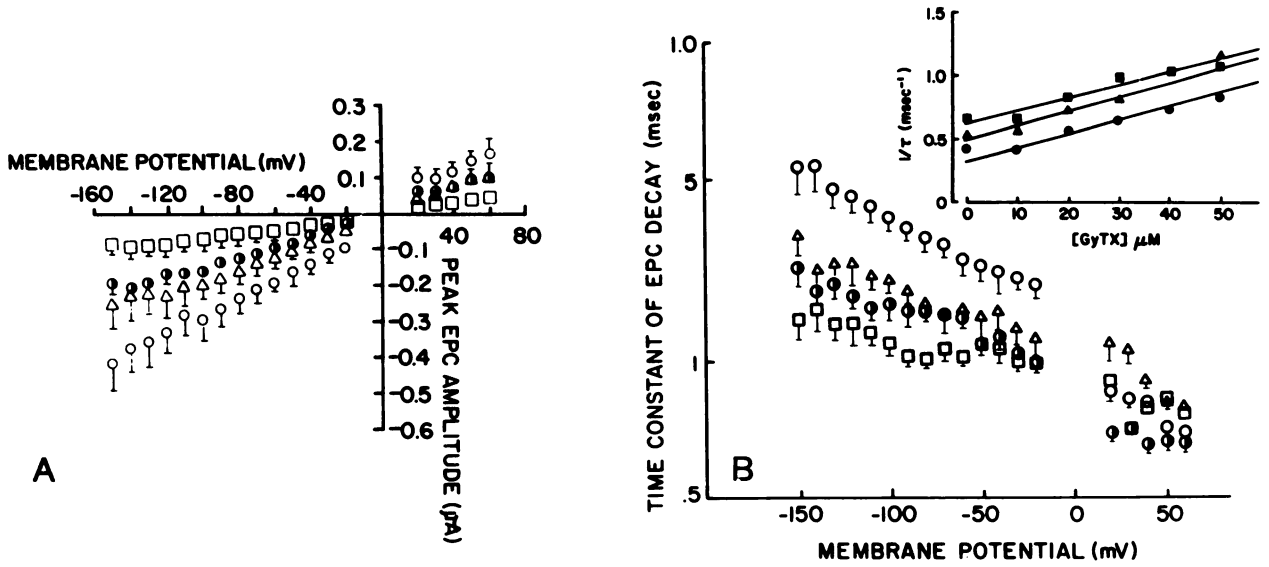

FIG. 4. *Current-voltage relationship of the EPC peak amplitude (A) and semilogarithmic plot of τ_{EPC} against voltage (B) under control conditions (○) and after 30-min exposure of the sartorius muscle to various GyTx concentrations: 20 μM (△), 30 μM (◑), and 40 μM (□)*

The I-V relationships can be considered linear for all concentrations shown. *Inset* in B shows the inverse of the decay time constant ($1/\tau_{EPC}$) against GyTX concentration at clamp potentials of −150 mV (●); −90 mV (▲), and −50 mV (■). The relationship is linear for all voltages. Each point represents the mean ± standard error of the mean of 18 junctional regions in 3 glycerol-treated muscles for each concentration of GyTX.

direction of the voltage excursion (Fig. 6). The results for τ_{EPC} were in agreement with the finding of almost no time-dependent effect of GyTX. Thus, at −90 mV and in the presence of 30 μM GyTX, τ_{EPC} was the same, independent of the direction of the voltage excursion. With 3-sec conditioning pulses, τ_{EPC} values were 3.99 ± 0.47 msec and 3.56 ± 0.3 msec; with 20 msec conditioning pulses, the values were 4.10 ± 0.52 msec and 4.40 ± 0.33 msec for the hyperpolarizing and depolarizing directions, respectively.

Effect of GyTX on EPCs induced by repetitive nerve stimulation. The effect of some compounds such as HTX (26), perhydrohistrionicotoxin (31), and meproadifen (13) seems to be enhanced upon previous stimulation of the preparation. This effect could be due either to an interaction of the compound with the ion channel of the AChR complex in its open configuration and/or an enhancement of desensitization (14).

To determine whether such an effect is present during exposure to GyTX, trains of 50 EPCs were elicited at 25,

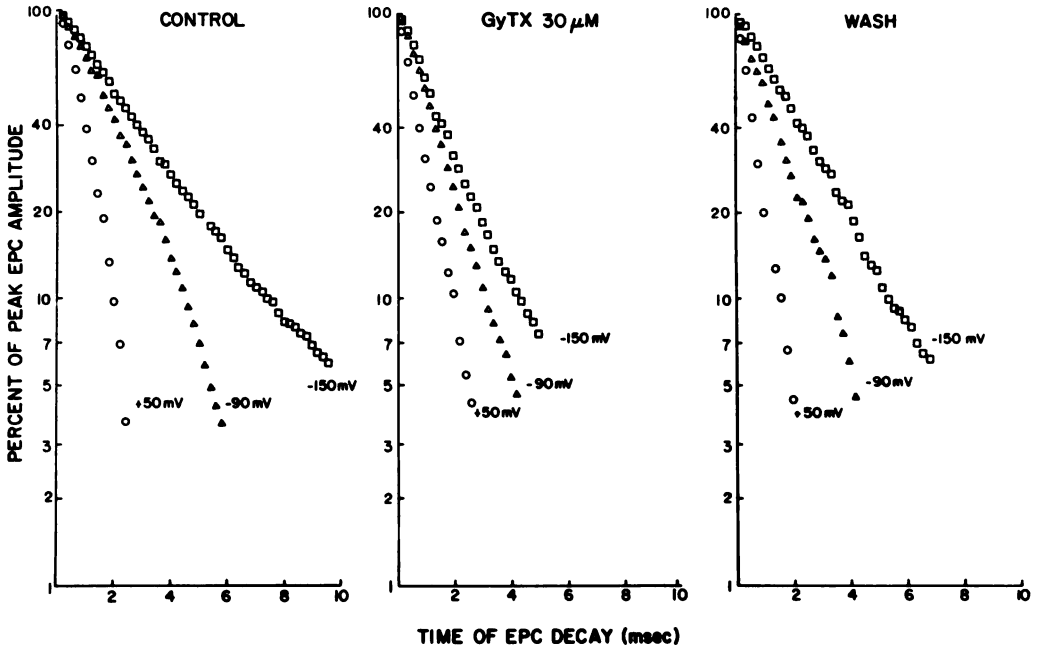

FIG. 5. *Effect of GyTX on the decay phase of EPC at three voltage clamp potentials (□, −150 mV; △, −90 mV; ○, +50mV)*

The percentage of peak EPC amplitude is plotted as a function of time in milliseconds. This is a representative sample showing control conditions, 30 min after treating the preparation with GyTX (30 μM) and after washing with GyTX-free physiological solution for 60 min. The decay phase of EPC remained a single exponential function of time under all conditions.

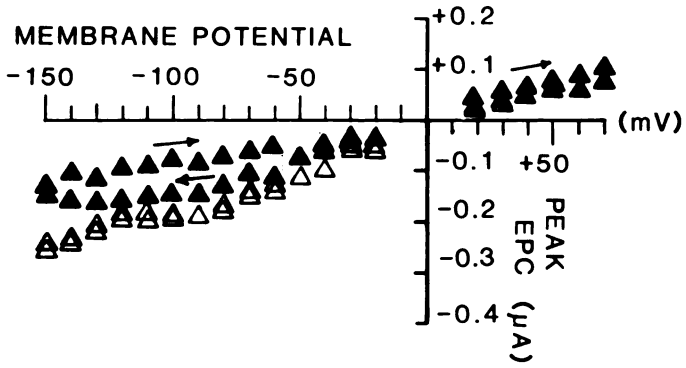

FIG. 6. *Effect of GyTX (30 μM) on the shape of the I-V relationship of peak EPC amplitude using conditioning pulses with 3-sec (△) and 20-msec (▲) duration*

This is a representative experiment and discloses no significant evidence of "hysteresis loop" or time-dependent effect of the alkaloid beyond that seen with control preparations.

50, and 100 sec^{-1}, at intervals of 2 min between each train. Typical records of EPC trains elicited at 25 sec^{-1} at various membrane potentials in the presence and absence of GyTX (20 μM) are shown in Fig. 7. After exposure to GyTX (20 μM), the peak amplitude of the first EPC of a train induced at 25 sec^{-1} and −90 mV was reduced by 73% in comparison to the first EPC of an equivalent train elicited in control condition. At stimulation frequencies of 50 and 100 sec^{-1}, an equal concentration of GyTX at −90 mV reduced the first EPC amplitude by 76% and 84%, respectively, in comparison to the first EPC in control cells. Two main features are evident from Fig. 7: (a) at positive potentials (+50 mV in this case), where the EPCs had a faster time course (τ_{EPC} is around 0.7 msec), previous stimulation had only a minor effect on peak EPC amplitude in the presence of GyTX; (b) at more negative potentials, the peak amplitudes of the EPCs were progressively decreased as they were elicited in a train. This rundown in EPC amplitude was also seen for other frequencies of stimulation, as shown in Fig. 8. For example, in the presence of 20 μM GyTX, the peak amplitude of the last EPC of a train induced at 25 Hz for 1 sec and −90 mV clamp potential was reduced to 42%. Likewise, in trains elicited at 50 and 100 Hz, the peak amplitudes of the last EPCs were reduced to 35% and 20% of control values, respectively. Figure 8 (*inset*) also shows that the frequency of stimulation does not affect τ_{EPC}. As can be seen, at −90 mV, GyTX depressed τ_{EPC}, as expected from the previous results, but τ_{EPC} maintained the same value regardless of the frequency of stimulation.

Effect of GyTX on the quantal content and quantal size of the EPP. Since the decrease in EPC peak amplitude enhanced by GyTX could be related to a presynaptic effect by GyTX decreasing the release of ACh, we measured the quantal content and quantal size of EPPs in the presence of high Mg^{2+} concentrations (for details see Methods). The results (Table 4) show that, even after 120 min of exposure to 20 μM GyTX, the EPP amplitude was not different from control. The quantal content was significantly increased, despite the fact that the quantal size had become smaller. This increase in quantal content is most likely due to the ability of GyTX to block potassium conductance (g_K) (see Figs. 2 and 3), a phenomenon similar to that observed with another g_K blocker, phencyclidine (34). Thus, instead of decreasing the amount of released ACh, GyTX actually tended to increase it.

Noise analysis of EPCs induced by iontophoretically applied ACh. The above results with GyTX on the end-plate point to an effect of the alkaloid on the ionic channel of the AChR complex, possibly blocking it while in the open conformation. If a simple sequential model for this kind of blockade is assumed (see, for example, refs. 12, 27, 33, 35, and 36), then the effect of GyTX should be manifest much more in terms of channel lifetime than conductance. In fact, this kind of behavior

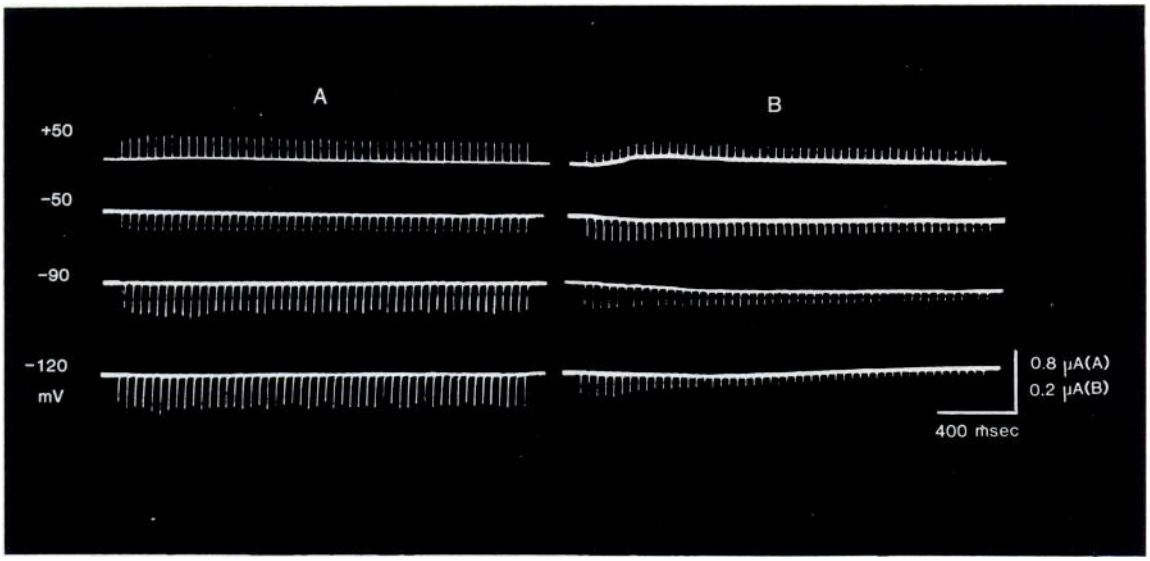

FIG. 7. *EPCs evoked by repetitive stimulation (25 Hz) (A) before and (B) after 30 min of exposure to GyTX (20 μM)*

The vertical scale shows current calibration for (A) control and (B) after GyTX. Horizontal scale is time in milliseconds. The numbers in the *left* of each trace indicate the holding potential.

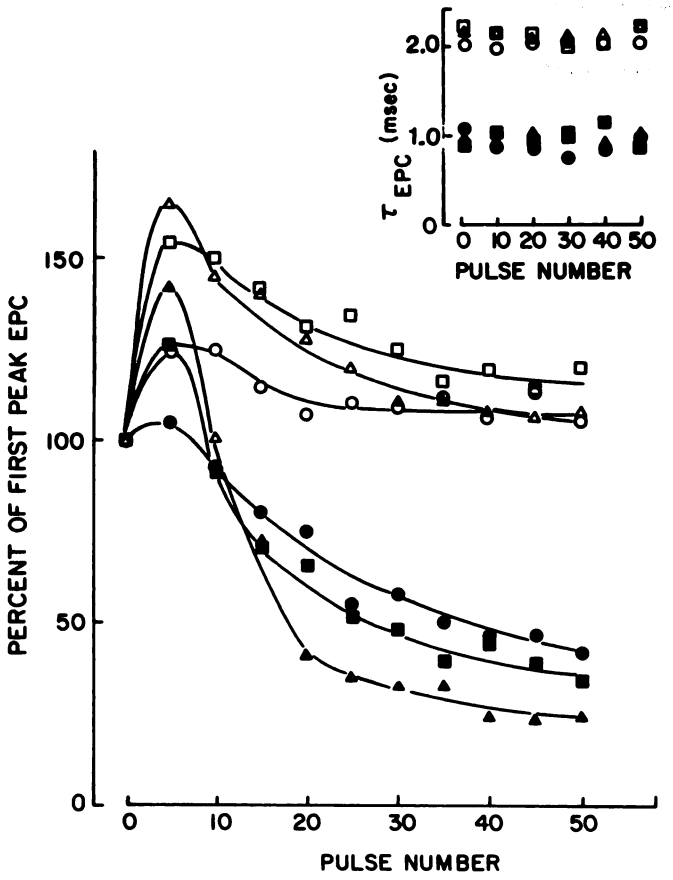

FIG. 8. *EPC peak amplitude during repetitive stimulation before (open symbols) and after 30 min of exposure to GyTX (20 μM; closed symbols)*

The EPC peak amplitude as a percentage of the amplitude of the first peak is plotted against pulse number for trains of EPC evoked at 25 Hz (○, ●), 50 Hz (□, ■), and 100 Hz (△, ▲). Holding potential was −90 mV under all conditions. Each point represents the mean of at least six determinations in the particular frequency used. For simplicity, only the mean values were used. The *inset* shows τ_{EPC} before (*open symbols*) and after GyTX (*closed symbols*) against pulse number. Note that τ_{EPC} did not change as a function of the frequency of activation but only as a function of GyTX presence.

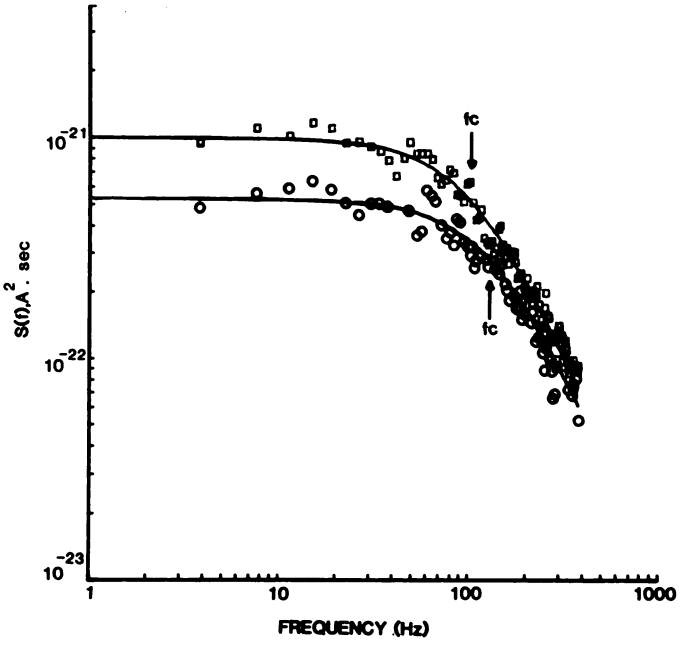

FIG. 9. *Power density spectra for microiontophoretically induced EPCs in frog sartorius muscle end-plate in control (□) and after treating the preparation for 30 min with 7.5 μM GyTX (○)*

Holding potential was −85 mV in both cases. Curves represent the best fit to a single Lorentzian. *Arrows* indicate the half-power frequency (*fc*) for control (109.98 Hz) and in presence of the toxin (140.7 Hz). γ and τ_I were 31.8 pS and 1.45 msec for control and 27.9 pS and 1.13 msec in presence of GyTX, respectively.

TABLE 4

Effect of GyTX on the amplitude, quantal size, and quantal content of the EPP in surface fibers of frog sartorius muscles

Numbers are expressed as percent of control obtained from 14 fibers of at least 5 sartorius muscles. For each junctional region, only a stable quantal content recorded 25–30 min under each condition was used for study of the effect of GyTX. A 60-min wash of the preparation started after 120 min of exposure to 20 μM GyTX. Results are expressed as means ± standard error of the mean.

Condition	Period of exposure	EPP amplitude	Quantal size	Quantal content
	min			
Control	0	100 ± 5	100 ± 4	100 ± 10
GyTX, 20 μM	30	126 ± 28	92 ± 8	135 ± 25
	60	115 ± 30	77 ± 10[a]	152 ± 49
	90	107 ± 20	73 ± 7[a]	156 ± 28[a]
	120	100 ± 12	66 ± 3[a]	155 ± 24[a]
Wash	—	88 ± 10[a]	86 ± 4[a]	101 ± 12

[a] Significantly different from control ($p < 0.05$).

TABLE 5

Effect of GyTX on single-channel conductance and single-channel lifetime in the frog sartorius muscle end-plate at 22°

Results were obtained during 30- to 60-min exposure to GyTX or after a 120-min wash of the preparation treated with 7.5 μM GyTX for 40 min. Values are means ± standard error of the mean of spectra obtained from 5–10 cells.

Condition	No. of spectra	Holding potential	Channel conductance	Channel lifetime
		mV	*pS*	*msec*
Control	17	−75	27 ± 1.6	1.05 ± 0.02
	25	−85	24 ± 1.0	1.28 ± 0.03
	11	−105	22 ± 1.4	1.60 ± 0.10
GyTX, 2.5 μM	7	−75	25 ± 2.2	1.01 ± 0.09
	10	−85	26 ± 1.6	1.28 ± 0.05
	8	−105	26 ± 2.1	1.60 ± 0.09
GyTX, 5.0 μM	6	−75	27 ± 3.2	1.04 ± 0.10
	12	−85	24 ± 1.7	1.21 ± 0.07
	6	−105	28 ± 2.2	1.39 ± 0.06[a]
GyTX, 7.5 μM	7	−75	31 ± 3.1	0.96 ± 0.07[a]
	7	−85	28 ± 2.5	1.15 ± 0.05[a]
	5	−105	25 ± 2.8	1.11 ± 0.15[a]
Wash	5	−75	28 ± 2.7	1.03 ± 0.14[a]
	7	−85	27 ± 2.7	1.03 ± 0.09[a]
	5	−105	19 ± 2.3	1.18 ± 0.24[a]

[a] Significantly different from control ($p < 0.05$).

is exhibited by channels treated with QX-222, an open-channel blocker, as observed with the patch clamp technique (10). To test our hypothesis we measured single ACh channel conductance and lifetime using the noise analysis technique (22). Figure 9 shows a power density spectrum for noise generated by microiontophoretically applied ACh in control and after 5 μM GyTX. In both cases the points could be fitted with a single Lorentzian, but the half-power frequency was increased upon exposure to GyTX. Table 5 shows these parameters more clearly. Channel conductance (γ) was not changed by GyTX (7.5 μM), although channel lifetime (τ_I) decreased by about 30% at −105 mV. It also should be noted that the action of the alkaloid on channel lifetime was voltage-dependent; at −105 mV, GyTX shortened channel lifetime more than at −75 mV, thus indicating a preferential action of GyTX on the open state of the AChR.

DISCUSSION

A previous report showed that GyTX had antimuscarinic effects on guinea pig ileum and atrium preparations (37). The present findings demonstrate that GyTX also blocks the neuromuscular transmission of the sartorius muscle of the frog, predominantly by affecting the ion channel of the AChR complex. Although GyTX has an action on the electrical excitability of the muscle membrane, prolonging the falling phase of the action potential (Figs. 2 and 3), through an effect on potassium channels, this action cannot explain the failure in neuromuscular transmission that follows GyTX exposure. In fact, a prolongation of the decay phase of the spike activity by GyTX would tend to facilitate neuromuscular transmission by leading to an increased release of ACh from the presynaptic terminal. This facilitation is also seen in both direct and indirect elicited muscle twitches when low concentrations of GyTX are used. Qualitatively similar effects have been reported for HTX (2) and for phencyclidine (38). The interaction of GyTX with the AChR complex leads to neuromuscular blockade and is manifest in two apparently distinct effects: (a) the toxin causes a strong depression in both peak EPC and MEPC amplitudes and decay time constants (Tables 2 and 3) and shortens single channel lifetime as revealed by fluctuation analysis (Table 5); and (b) it induces an increase in the affinity of ACh for its binding site in the AChR complex [discussed in the companion paper (14)]. In fact, Souccar *et al.* (26) have shown, using the patch clamp technique on cultured rat myoballs, a reduction in single-channel lifetime upon addition of GyTX to the solution inside the patch micropipette. Although the action of GyTX on channel lifetime was concentration-dependent, the toxin had no effect on single-channel conductance. Thus, one of the actions of GyTX seems to be blockade of the ion channels in open conformation. A simple sequential model (8, 10, 11 35, 39) can suffice to explain our results as follows:

$$n\mathrm{ACh} + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ACh}_n\mathrm{R} \underset{k_{-2(v)}}{\overset{k_{2(v)}}{\rightleftharpoons}} \mathrm{ACh}_n\mathrm{R}^* \underset{k_{-3(v)}}{\overset{\overset{D}{k_{3(v)}}}{\rightleftharpoons}} \mathrm{ACh}_n\ \mathrm{R}^*D$$

where n is the number of ACh molecules, R is the receptor channel complex; $\mathrm{ACh}_n\mathrm{R}$ is the activated receptor, $\mathrm{ACh}_n\mathrm{R}^*$ is the open conformation of the channel, $\mathrm{ACh}_n\mathrm{R}^*D$ is the open but blocked conformation, and D stands for the blocker. The k's are rate constants for the reactions indicated; those followed by (v) are supposed to be voltage-dependent. Here we assume that the formation of $\mathrm{ACh}_n\mathrm{R}^*$ has been completed when the peak of the EPC is reached. Therefore, the rate constants k_1, k_{-1}, and $k_{2(v)}$ need not be considered in this case.

As expected from this model, the main effect of an open blocker should be on the channel lifetime (τ_I); i.e., in the presence of the drug, the open channels would tend to enter a blocked pool upon binding to drug molecules and thereby have a shortening in their lifetime. GyTX has exactly this kind of effect, both as evidenced by the single-channel lifetime (Table 5) as measured by fluctuation analyses and by the τ_{EPC} (Table 3; Fig. 4B). The effect on τ_{EPC} could also be the major factor responsible for decreasing peak EPC amplitude (Fig. 4A). The basic assumption here is that the blocking reaction is fast enough (k_3 is large) so that, before all channels are open, some of them have already entered the blocked pool. This kind of phenomenon has been seen with atropine (8). On the other hand, drugs such as HTX (2, 6), and meproadifen (13), whose action is primarily on the closed-channel conformation, have little or no effect on τ_{EPC} but greatly depress peak EPC amplitude, disclosing a nonlinearity in the third quandrant of its I-V relationship. With GyTX the I-V plot for peak EPC amplitude is almost completely linear (Fig. 4A). The slight nonlinearity is also seen under control conditions (31). In keeping with this linearity in the I-V relationship, there is an absence of a considerable time-dependent effect (see Results and Fig. 6), in contrast to what is seen with HTX (6) and meproadifen (13).

From the model we also expect a linear relationship between $1/\tau_{EPC}$ and GyTX concentration; i.e., $1/\tau_{EPC} = k_{-2} + [D]k_3$ (assuming $k_3 \gg k_3$). Such a relationship is clearly seen in Fig. 4B (*inset*) for concentrations of GyTX up to 50 μM. Such linearity has been described also for phencyclidine methiodide and piperocaine methiodide (30), QX-222 (10), procaine (35), and others, in sharp contrast to the saturating behavior observed with perhydrohistrionicotoxin (2).

The effect of temperature on τ_{EPC} at −90 mV (Table 3) also favors an open block mechanism for GyTX. Decreasing the temperature to 10° greatly increases τ_{EPC} (10.55 msec), which means a larger lifetime for the channels. Under this circumstance, τ_{EPC} decreases about 64%, as opposed to only a 34% decrease at 22° (values refer to a GyTX concentration of 30 μM).

Another action of GyTX is the so-called use-dependent effect, as shown in Figs. 7 and 8. This kind of behavior can be predicted from the model above if one assumes that the blocking reaction is slowly reversible (i.e., $k_3 >> k_3$) (40). Thus if several attempts are made to open channels in a short period of time, an increasingly larger fraction of these channels will tend to enter the blocked state. The assumption of $k_3 >> k_{-3}$ is supported by the fact that only a single exponential decay is seen for the EPCs in the presence of GyTX (Fig. 5). A fast dissocia-

tion of the blocker would result in the presence of transiently open channels, and a slow tail would be seen in the EPC decays, as reported for scopolamine (8) and QX-222 (9, 11). Such an effect is not seen with GyTX.

The model also permits a rough estimation of the fraction of the electrostatic potential sensed by GyTX at its binding site by looking at the voltage dependence of k_3 (41). The basic data come from results as shown in Fig. 4B (*inset*). From the slope of the lines relating $1/\tau_{EPC}$ to GyTX concentration at various clamp potentials, we can obtain k_3 as a function of voltage. The small differences in these slopes (Fig. 4B, *inset*) indicate that k_3 for GyTX blockade of the open channel is slightly voltage-dependent. Such finding could have two interpretations: either (a) GyTX does not have to penetrate appreciably into the channel to cause blockade, or (b) the active form of the alkaloid is uncharged. Our data do not permit distinction between these two possibilities. However, at pH 7.2, GyTX would be present mostly in its cationic form, since the pK_a for such a tertiary amine of this structure can be estimated to be greater than 9. An external binding site has been proposed for phencyclidine methiodide (the fraction of the transmembrane electric field that the compound has traversed at its rate-limiting energy barrier was on the order of 6%; see refs. 30 and 38), in contrast to QX-222 (10), QX-314 (42), and procaine (35), which appear to have to penetrate the channel to a greater depth in order to cause a blockade.

In conclusion, the results presented here can be adequately explained by assuming that GyTX blocks neuromuscular transmission by interacting with the AChR complex so as to block the channel while in its open configuration.

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