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Mechanism of Action of Amantadine on Neuromuscular Transmission

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SUMMARY

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The effect of amantadine on neuromuscular transmission was studied in frog sartorius and rat soleus and diaphragm muscle preparations. Amantadine blocked transmission reversibly, while having negligible presynaptic effects and inducing 10–20 mV depolarization of the muscle membrane that was tetrodotoxin insensitive. Its most pronounced effect was on the acetylcholine receptor-mediated postsynaptic conductances. Amantadine caused voltage-dependent attenuation of the peak endplate current amplitude and marked departure from linearity in the current voltage relationship. The drug altered the voltage-dependence of the falling phase of the endplate current, and reduced the slope of the relationship between endplate current half decay time and membrane potential with subsequent reversal of the slope such that the endplate current became faster with hyperpolarization. In addition, amantadine was found to inhibit the carbamylcholine-induced ^{22}Na efflux from microsacs formed from *Torpedo* electric organ membranes, suggesting that amantadine had postsynaptic action. Amantadine did not inhibit binding of [^{3}H]acetylcholine or [^{125}I]α-bungarotoxin to the acetylcholine receptor but inhibited competitively the binding of [^{3}H]perhydrohistrionicotoxin to the ionic channel of the acetylcholine receptor with K_i of 60 μM. These effects of amantadine on postsynaptic ionic current, coupled with its inability to protect against blockade of transmission by α-bungarotoxin and its inhibition of [^{3}H]perhydrohistrionicotoxin binding suggest that amantadine blocks neuromuscular transmission by reacting with the ionic channel of the acetylcholine receptor.

INTRODUCTION

In the last five years there has been considerable progress towards elucidating the mechanisms underlying the interaction of ACh[1] with its recognition site in the postsynaptic membrane and the subsequent and less understood events, which lead to an increase in the transient conductance changes in the membrane, now thought to involve the complex of the receptor and its ionic channel[2] (1–5).

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[1] Abbreviations used: ACh, acetylcholine; HTX, histrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin; DFP, diisopropylfluorophosphate; EDL, extensor digitorum longus; epc, endplate current; epp, endplate potential; mepp, miniature endplate potential.

[2] The term "ion conductance modulator" has been routinely used by our laboratories (1, 2, 11). We are using the term "ionic channel" instead for the sake of simplicity.

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In addition to α-bungarotoxin (6), a specific probe for the neuromuscular nicotinic ACh receptor, another group of natural toxins, HTX and its analogues, isolated from the skin secretions of the arrow poison frog *Dendrobates histrionicus,* were introduced as specific probes for the ionic channel of the ACh receptor (7–10). The binding of the tritiated and totally reduced form of HTX, H_{12}-HTX, was used to identify the ionic channel in membrane preparations from *Torpedo* electric organs (11–13). Competitive antagonists like *d*-tubocurarine and α-bungarotoxin are believed to react with the ACh receptor at concentrations that do not affect the elementary current generated by ACh (13–15). They reduce the amplitude of the epc but do not alter its rate of decay. Only higher concentrations of *d*-tubocurarine (>6 μM) affect the ionic channel (Albuquerque & Eldefrawi, unpublished results). HTX, its analogues, and antimuscarinic drugs such as atropine, shorten the elementary process induced by ACh, and reduce the amplitude and decay time of the epc. In addition, these agents reduce the voltage sensitivity of the decay in a dose-dependent manner (2, 16, 17).

Amantadine hydrochloride (1-adamantanamine hydrochloride, Symmetrel) is an antiviral drug known for its prophylactic effect against A_2 (Asian) influenza in animals and man (18). The apparent antiviral activity results from the ability of the drug to prevent viral penetration into the host cell. In addition, amantadine is also relatively effective in the treatment of human Parkinsonism (19). Although it is considered likely that amantadine exerts its antiparkinson effect through an action similar to that of L-dopa (20), the drug has recently been shown by Nastuk *et al.* (21) to block neuromuscular transmission by reducing the response of muscle postjunctional membrane to ACh. The present study, utilizing electrophysiological as well as biochemical techniques, was initiated to determine whether the reaction sites for amantadine at the postsynaptic membrane were on the ACh receptor or its ionic channel or both.

METHODS

Animals and preparations. Experiments were carried out at room temperature (22–24°C) on sartorius muscle preparations of the frog, *Rana pipiens,* and soleus and diaphragm muscles of the rat (Wistar females, 180–200 g). ACh sensitivity determinations were made using the chronically denervated (14–17 days) soleus muscle of the rat. Binding studies were done on receptor-enriched membrane preparations from the electric organ of *Torpedo ocellata.*

Solutions. During dissection and throughout the electrophysiological experiment the muscles were bathed in a physiological solution having the following composition (mM concentration): NaCl, 115.5; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4, 1.3; NaH_2PO_4, 0.7 for frog muscles, and NaCl, 135.0; KCL, 5.0; $CaCl_2$, 2.0; $MgCl_2$, 1.0; $NaHCO_3$, 15.0; NaH_2PO_4, 1.0; and glucose, 11.0 for mammalian muscles. For frog muscles the pH of the solutions was 6.9–7.1, and they were bubbled with 100% O_2. Mammalian physiological solutions were continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 which gave them a pH of 7.1–7.3. When indicated, the calcium concentration of the frog Ringer's solution was raised from 1.8 to 15 mM. In voltage clamp experiments, the muscles were treated with a hyperosmotic solution of glycerol in normal frog physiological solution until the indirectly elicited twitches were blocked (22). The muscles were pretreated with 400–600 mM glycerol to disconnect functionally the sarcotubular system from the sarcolemmal membrane and allow the superimposition of several action potentials without causing contraction of the muscle fibers.

Drugs. The following drugs were stored as refrigerated stock solutions; they were diluted with physiological solution immediately before use. The stock solutions were as follows: amantadine hydrochloride (DuPont), *d*-tubocurarine chloride (K & K Laboratories), 10^{-2} M and tetrodotoxin (Sankyo Co.), 3×10^{-4} M in distilled water. α-Bungarotoxin was purified by ion exchange chromatography on Sephadex CM50, and the stock solution in 5 mM Na_2HPO_4, pH7, was stored at −20° and used within two months.

[*acetyl*-^{3}H]Acetylcholine (sp. act. 49.5 Ci/mole) and ^{22}NaCl (carrier free, 2.5 Ci/ml) were obtained from New England

Nuclear and [^{3}H]H_{12}-HTX (sp. act. 4.8 Ci/mole) was obtained by tritiation of isodihydrohistrionicotoxin and its activity tested on frog sartorius muscles as described (10). α-Bungarotoxin was iodinated by the chloramine-T method as described (23).

Electrophysiological Techniques. The muscles were dissected during superfusion with Krebs-Ringer physiological solution. For *twitch tension* studies, the left phrenic nerve was stimulated with supramaximal pulses having a duration of 0.2 msec via a Ag-AgCl salt bridge electrode connected to a wet electrode (24). The stimulation frequency was 0.05 Hz. Direct stimulation was obtained by applying supramaximal rectangular pulses of 1.0–2.0 msec duration at a rate of 0.05 Hz through a bipolar platinum electrode placed at the base of the diaphragm muscle near the intercostal muscles. The twitch tension was recorded by attaching the muscles to a Grass F. T. 03 force displacement transducer and by displaying the twitch on a Grass polygraph. At least 30 min was allowed for muscle stabilization in the chamber.

For *intracellular recordings,* the muscles were stretched slightly beyond their resting length in a Plexiglas plate, which had a planoconvex lens at its center, and immersed in a 15 ml bath. Nerve stimulation was delivered by two platinum electrodes. All recordings were made from surface fibers using glass microelectrodes filled with 3 M KCl (5–15 MΩ). Microelectrodes that developed tip potentials larger than 1–3 mV were rejected and the muscle fiber discarded. The potentials were displayed on a Tektronix oscilloscope and recorded on a Mingograph 81. Data from the oscilloscope were recorded on film and measured under magnification.

Membrane excitability was studied in the frog with standard microelectrode techniques (25). The threshold depolarization for the generation of the action potential was taken as the amplitude of the potential step preceding the self regenerative response. The amplitude of the potential between threshold and summit. The repolarization phase was represented by the time of half decay of the total membrane depolarization. The maximum rate of rise of the action potential (dV/dt) was measured by means of an RC circuit (100 K Ω-100 pF) and was recorded on the oscilloscope.

The *electrical constants of the membrane* were measured by inserting two micropipettes into the surface fibers of the frog sartorius muscle. A rectangular hyperpolarizing pulse of 150 msec duration was passed through one microelectrode, and the change in membrane potential was recorded with the other micropipette. Measurements were made at three interelectrode distances: 0.05–0.1; 0.3–0.4; and 0.5–1.0 mm. Fibers that became depolarized by more than 10 mV during the course of these measurements were discarded. The membrane constants were calculated by applying the cable theory of Hodgkin and Rushton (26) and the square pulse analysis of Fatt and Katz (27) as described by Albuquerque and McIsaac (28).

Endplate potentials (epp) were monitored on a Tektronix 565 oscilloscope and sampled at 100 μsec intervals by a PDP 11/40 on line computer. Two hundred potentials were recorded from each cell, and the amplitude of each was determined by the computer. Care was taken to see that electrical artifacts or noise points were not mistaken by the computer for the peak of the potential. The first ten potentials were discarded, and the next nine groups of 20 potentials were used to obtain estimates of the quantal content and size as described previously (29).

The *voltage clamp* circuit used was similar to that previously described (30, 31). Voltage clamp errors were less than 5% of the unclamped epp. The time constant of the clamping circuit (10–90%) with 3–8 MΩ microelectrodes was less than 20 μsec. Changes in membrane potentials from the holding potentials were evoked either from a DC source under manual control or an isolated stimulus unit controlled by a programmable digital timer (8, 11). Waveforms were recorded on film from the display of a 565 Tektronix oscilloscope, or sampled by the PDP 11/40 computer.

Biochemical techniques. Membranes were prepared from the electric organ of *T. ocellata* (stored at −90°C) by homogenization (20%, w/v) in ice cold solution of 90 mM KCl, 10 mM NaCl, and 1 mM Na_2HPO_4

(pH 7.4). The supernatant of a 5,000 × *g*, 10 min centrifugation was recentrifuged at 30,000 × *g*, 60 min. The pellet was resuspended in Krebs original Ringer phosphate solution (NaCl, 107 mM; KCl, 4.8 mM; $CaCl_2$, 0.65 mM; $MgSO_4$, 1.23 mM; Na_2HPO_4, 15.7 mM; pH, 7.4) (32) (1 ml representing 1 g of the electric organ), and the membranes formed microsacs. Maximum number of binding sites for [^{3}H]ACh and [^{3}H]H_{12}-HTX were 0.7 and 0.5 nmoles/mg protein, respectively.

Binding was determined by equilibrium dialysis at 21°C for 4 hr as previously described (10). One half milliliter of membrane preparation was placed in a pretreated dialysis bag (33) which was tied at both ends and immersed in 25 ml bath of Krebs original Ringer phosphate solution containing the radioactive ligand, then shaken for 4 hr. Triplicate samples of 50 μl each were then taken from each dialysis bag and their radioactivity counted in 5 ml of toluene scintillation solution. Excess radioactivity per unit volume of bag content over bath samples represented the amount of ligand bound. When binding of [^{3}H]ACh was studied, DFP was added to the membranes or extract at a final concentration of 1 mM one hour before the start of dialysis, and 100 μM of DFP was present in the dialysis bath. At these concentrations, DFP inhibited all cholinesterases present without affecting ACh binding to the ACh receptor. When the effect of drugs on [^{3}H]ACh or [^{3}H]H_{12}-HTX binding was determined, the drug was placed only in the dialysis bath except in the case of α-bungarotoxin, which was placed with the receptor preparation one hour before dialysis.

Binding of [^{125}I]α-bungarotoxin was measured by a filter assay using Whatman GF/C fiber glass filters (details of the procedure will be published elsewhere).

Studies of ^{22}Na efflux were conducted on microsacs, using a method modified from Hess *et al.* (34). In this method carbamylcholine was added only after most of the nonspecific ^{22}Na efflux ended and a steady state was reached; thus interference of nonspecific efflux (35, 36) was avoided. To each 1 ml of microsac preparation (containing 0.02% NaN_3), 25 μl of ^{22}NaCl was added and incubated at 1–2°C for 36 hr. A sample of 100 μl was then diluted with 20 ml ice cold solution of 250 mM KCl, 5 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM Na_2HPO_4, pH 7. Two 1 ml samples were taken at 0 time and after 30, 35, 40, 42.5, 45, 50, 60 and 65 min. Each sample was filtered on a HAWP 0.45 μm Millipore filter and rinsed twice with 10 ml ice cold solution of 5 mM KCl, 250 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM Na_2HPO_4, pH 7, then the radioactivity in the filters counted in an auto-gamma scintillation spectrometer (Packard 5230). At 35 min, the remaining preparation was split into two portions, one acting as control for this experiment having no drugs added, while the second was exposed to a drug or toxin at 36 min. Having this internal control for each run reduced greatly the variability that was found between different membrane preparations. At 39 min, carbamylcholine was added at a final concentration of 100 μM.

Acetylcholinesterase activity was assayed by the Ellman *et al.* (37) spectrophotometric method.

RESULTS

Effects of the combination of α-bungarotoxin and amantadine on neuromuscular transmission and endplate potentials of the phrenic-nerve diaphragm muscle preparation of the rat. The dose-effect relationship of amantadine (1×10^{-4} to 5×10^{-2} M) on the rat diaphragm muscle preparation is shown in Fig. 1. Prior to block of neuromuscular transmission there was an initial potentiation of the indirectly elicited muscle twitch. This effect was most evident with concentrations of amantadine ranging from 1.5×10^{-4} to 5×10^{-4} M (Fig. 1). It was absent with much higher concentrations of amantadine, which was most likely related to the very fast rate of neuromuscular block.

The effect of a high concentration of amantadine (2×10^{-3} M) on the phrenic nerve-diaphragm muscle preparation of the rat shows that the onset of drug effect on the indirectly elicited muscle twitch occurred within 2 min; by 5 min the twitch was 50% blocked, and in 10 min complete block occurred. The simultaneously re-

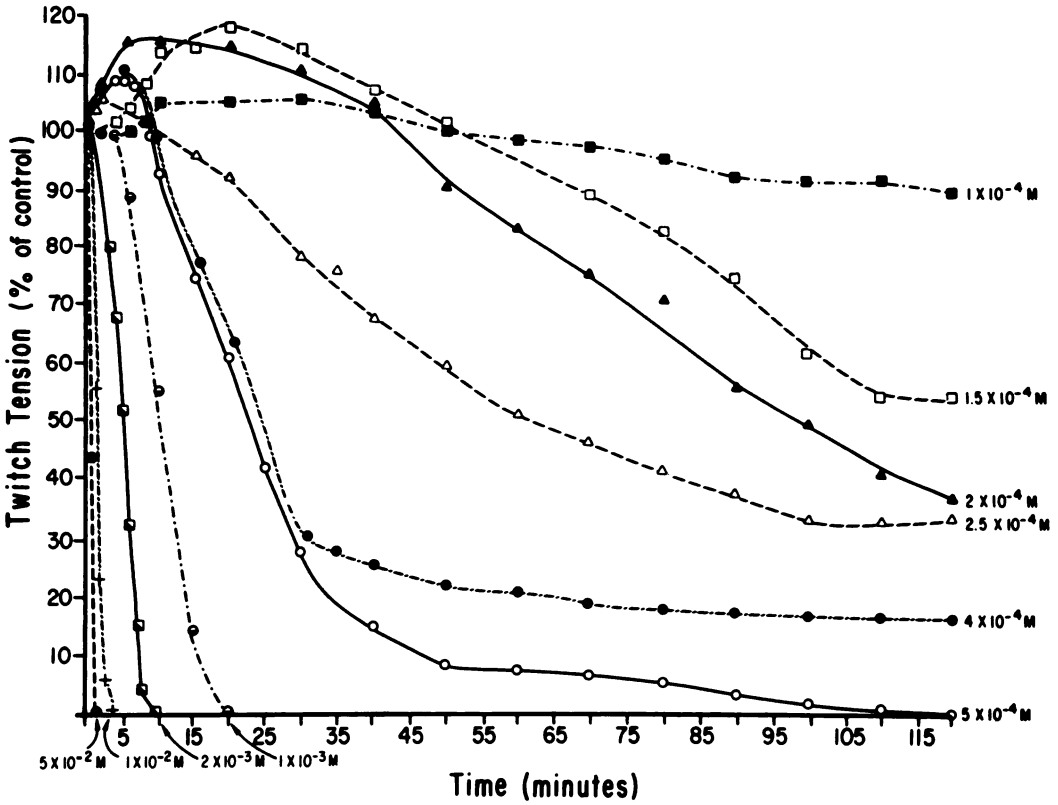

FIG. 1. *Time course of block of the indirect twitch of the rat phrenic nerve diaphragm preparation produced by different concentrations of amantadine recorded at the temperature of 36°C.*

corded, directly elicited muscle twitch was only slightly affected. Although the drug was effective at this concentration in blocking muscle twitch, washing of the muscle with Krebs-Ringer solution caused complete dissociation of the drug from its reactive sites in 120–150 min. At this concentration, amantadine (2×10^{-3} M) caused complete blockade of the epp (Fig. 2B). This block of neuromuscular transmission with amantadine suggests that the drug may have interfered either with the ACh receptor or its ionic channel. To distinguish between the two possibilities an analysis, similar to that made previously (9) on *d*-tubocurarine interaction with α-bungarotoxin, was made on amantadine interaction with α-bungarotoxin. Neuromuscular transmission was blocked in 10 min with 2×10^{-3} M amantadine, and all the epp's recorded at the surface fibers were blocked in 15 min. After 30 min exposure to amantadine the preparation was then exposed to a combination of amantadine (2×10^{-3} M) and α-bungarotoxin (5 μg/ml), the concentration of the latter being high enough to completely block the epp after 30 min of exposure (8, 9). Following 30 min of exposure to this combination of drugs, α-bungarotoxin was removed by washing the preparation for another hour with amantadine at the same initial concentration; the preparation then was washed with Krebs-Ringer solution for another 4 hr. Recordings of epp's 4 hr after washing disclosed that only a 0.5 to 1 mV epp was present (Figs. 2A-3), which did not differ from the amplitude obtained in absence of amantadine. This recovery of a small epp, which occurred after extensive washing, indicated that amantadine, at the high concentration used, was unable to protect ACh receptor against the almost irreversible action of α-bungarotoxin.

Effects of amantadine on the spontaneous and evoked transmitter release of

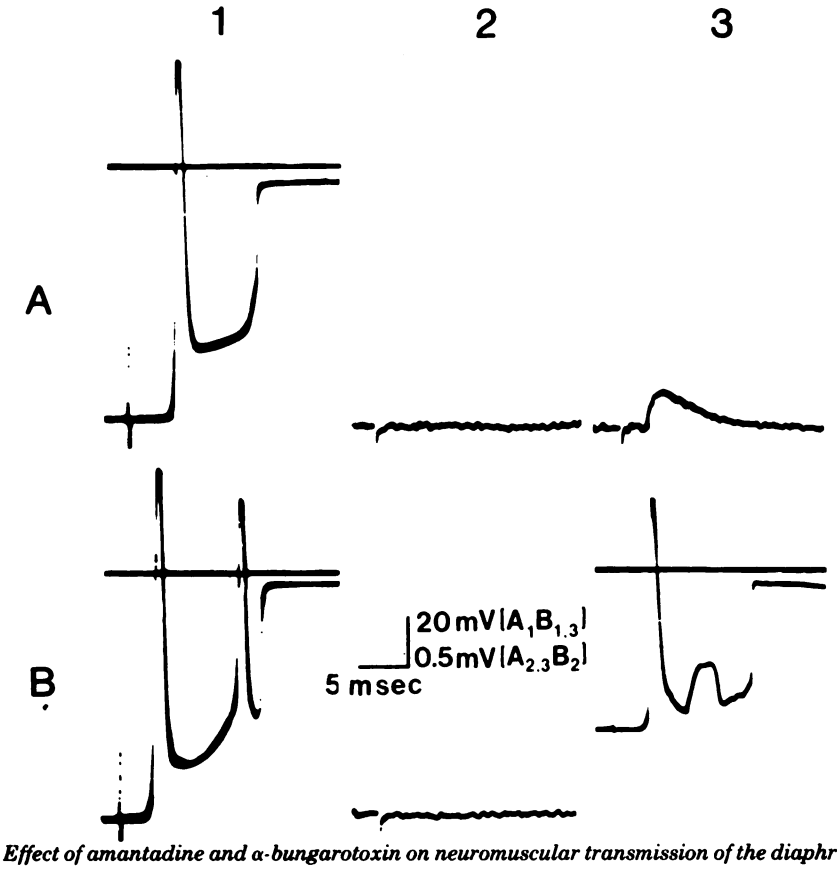

FIG. 2. *Effect of amantadine and α-bungarotoxin on neuromuscular transmission of the diaphragm muscle of the rat*

A-1 and B-1 are controls. A-2 is amantadine alone for 30 min (2×10^{-3} M), followed by the combination of amantadine and α-bungarotoxin (5 μg/ml) for 30 min, then subsequent wash with amantadine alone for another 30 min. The record is taken at the end of the second incubation period. B-2 is a record obtained after 90 min incubation with amantadine (2×10^{-3} M) alone. B-3 is a record obtained after washing the preparation for 120 min. A-3 and B-3 are at 240 and 120 min, respectively, after beginning to wash the preparation with normal Ringer's solution. Note that recovery of neuromuscular transmission is seen only when amantadine is used alone (B-3).

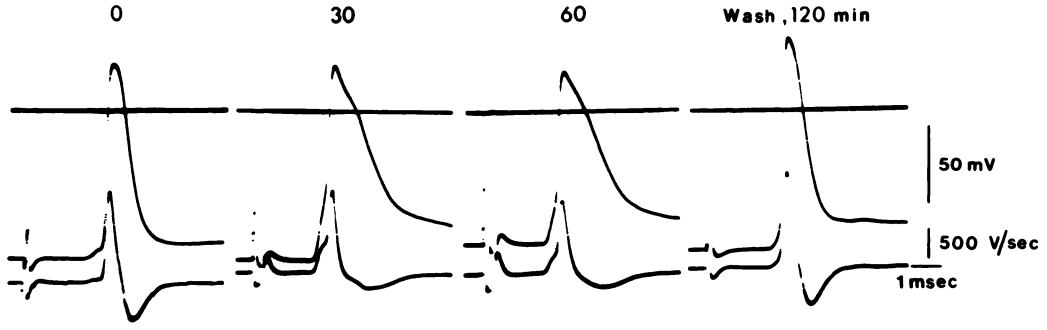

FIG. 3. *Indirect elicited action potentials recorded from glycerol shocked frog sartorius muscles before and at 30 and 60 min during exposure to amantadine (2×10^{-4} M)*

The last record is after 120 min of washing. Lower trace is the first derivative (dV/dt) of the action potential. Stimulation frequency is 1 Hz. Note that at 30 min even prior to membrane depolarization the action potential is already prolonged.

the sartorius muscle of the frog and rat diaphragm. Amantadine (2×10^{-4} M) did not affect quantal content of the epp, but it significantly decreased quantal size and frequency of the miniature endplate potential (mepp) of the frog sartorius muscle (Table 1). In fact, at this concentration, amantadine decreased the amplitude of the spontaneous mepp, and at 45 min of exposure to the drug, the potentials were too small to be recorded (Table 1). At lower concentrations (2×10^{-5} M), exposure to amantadine for 2 hr reduced the amplitude of mepp's to 40% of control. Similar results were also obtained in the rat diaphragm muscle. It should be mentioned that in spite of causing significant post-junctional membrane depolarization, the drug apparently did not depolarize the presynaptic nerve terminal, as revealed by its ineffectiveness in causing an increase in spontaneous mepp's. We may not be able, however, to exclude the possibility that amantadine partially increases transmitter release with a net result of no apparent change in mepp frequency. This finding is in marked contrast with that of tityustoxin (38) which produced a partial postsynaptic membrane depolarization and simultaneously induced marked depolarization of the presynaptic nerve terminal.

Effects of amantadine on junctional and extrajunctional sensitivity to acetylcholine of the chronically denervated mammalian muscles. Amantadine (10^{-4} M) markedly decreased the extrajunctional ACh sensitivity of the chronically denervated rat soleus muscle (Table 2), thus indicating that the drug had an affinity for the ACh receptor-channel complex. However, as observed with other drugs such as *d*-tubocurarine (8,

TABLE 2

Extrajunctional acetylcholine sensitivity of 14–17 days denervated rat soleus muscles

Values are expressed as the mean ± SEM of at least 25 single surface fibers of 3–5 muscles.

Amantadine	ACh sensitivity
	mV/nC
0	690 ± 77.8
1×10^{-4} M	183 ± 42.7[a]
2×10^{-4} M	92 ± 11.1[a]
1×10^{-3} M	69 ± 7.2[a]

[a] Results obtained at 45–60 min exposure to the drug.

TABLE 1

Effect of amantadine on the spontaneous miniature endplate potential amplitude and frequency as well as on the endplate quantal content and size of the frog sartorius muscles

Amantadine concentrations	Miniature endplate potential											
	Frequency (sec^{-1})[a] after different periods (min)						Amplitude (mV)[a] after different periods (min)					
	0	15	30	45	60	120	0	15	30	45	60	120
2×10^{-5} M	0.79 ± 0.16	0.59 ± 0.04	0.48 ± 0.05	0.48 ± 0.06	0.47 ± 0.06	0.11 ± 0.05	0.46 ± 0.03	0.41 ± 0.02	0.40 ± 0.08	0.38 ± 0.04	0.25 ± 0.03	0.20 ± 0.01
2×10^{-4} M		0.43 ± 0.12	0.16 ± 0.02	0.05 ± 0.01	—[b]	—[b]		0.14 ± 0.01	0.09 ± 0.01	—[b]	—[b]	—[b]
	Endplate potential[c]											
	Quantal content						Quantal size (mV)					
	0	15	30	45	60	120	0	15	30	45	60	120
2×10^{-4} M	60.21 ± 13.37				70.89 ± 15.32	81.31 ± 14.85	0.17 ± 0.01		0.05 ± 0.008		0.03[d] ± 0.005	0.02[d] ± 0.007

[a] m.e.p.p. frequency and amplitude were evaluated in 3–7 muscles of at least 75 single potentials. All values are mean ± SE.

[b] m.e.p.p. is too small to be recorded.

[c] endplate potential were recorded in the presence of Ringer solution containing 15 mM $MgCl_2$.

[d] $p < 0.01$ with respect to control (0 time).

39), amantadine was much less potent on the extrajunctional ACh receptors than on junctional ones. For example, the same concentration of amantadine that was effective in blocking the epp was unable to block the extrajunctional ACh sensitivity. This effect of amantadine on the ACh sensitivity appears to be dependent on the drug concentration, the length of exposure to the drug, and to a much lesser degree, upon the rate of stimulation used to apply ACh microiontophoretically onto the membrane.

Effects of amantadine on resting membrane potential and cable properties. Exposure of frog sartorius to amantadine (2×10^{-4} M) at room temperature for periods varying from 30 to 120 min produced significant depolarization of the frog sartorius muscle membrane (Table 3). The membrane depolarization caused by amantadine on rat diaphragm muscle occurred simultaneously with the development of muscle contracture, the latter attaining a maximal tension of 20 g. The membrane depolarization produced by amantadine was not prevented when the drug was used in combination with either low $[Na]_o$ (5 mM) Ringer's solution or tetrodotoxin (3×10^{-6} M) (Table 3). Indeed, a 28% membrane depolarization occurred, suggesting that the depolarization induced by the drug was not related to an increase in sodium conductance in either rat or frog muscle. Since amantadine, in very high concentrations (e.g., 1×10^{-2} M), also induced significant muscle contracture probably related to membrane depolarization, the possibility was raised that increased extracellular calcium concentrations might lower the level of membrane depolarization induced by amantadine. However, as shown in Table 3, 15 mM calcium in the Ringer's solution did not prevent the depolarizing action of amantadine.

Amantadine (2×10^{-4} M) caused no significant ($p < 0.01$) alterations of membrane electrical constants of the frog sartorius muscle, control value being 3919 ± 179 (mean ± SEM) and 60 min of exposure to the drug 3406 ± 437 for 12 fibers from three muscles.

Effects of amantadine on the action potential generating mechanism. Amantadine (2×10^{-4} M) prolonged the falling phase of the action potential in surface fibers of frog sartorius muscles recorded about 100–300 μm from endplate region (Fig. 3). This effect was observed when either single or repetitive action potentials were elicited. When the half decay time of the 5th and 10th elicited action potentials in repetitive stimulations were plotted against the concentration of amantadine, maximum prolongation of the action potential was elicited after 90–120 min of exposure. In addition, the amplitude and rate of rise of the action potential were also markedly decreased (Table 4). These changes in the action potential produced by amantadine were observed also in the rat diaphragm muscle. The prolongation of the duration of the action potential caused by amantadine suggests that the drug may have an effect on potassium conductance.

TABLE 3

Effect of amantadine on the resting membrane potential of frog sartorius muscle

Condition	Resting membrane potential (mV)[a]			
	Time (min) after amantadine application			Washing (60–90 min)
	0	60	120	
Amantadine (2×10^{-4} M)	−96.8 ± 0.9	−87.5 ± 1.6[b]	−66.8 ± 2.1[b]	−83.4 ± 2.8
Amantadine (2×10^{-4} M) & low Na^+ (5 mM)	−94.6 ± 0.6	−80.2 ± 1.5[b]	−67.8 ± 1.1[b]	−94.0 ± 1.9
Amantadine (2×10^{-4} M) & Tetrodotoxin (3×10^{-6} M)	−92.6 ± 1.3	−80.9 ± 1.5[b]	−67.2 ± 1.5[b]	−96.2 ± 1.2
Amantadine (2×10^{-4} M) & high Ca^{2+} (15 mM)	−101.2 ± 1.4	−85.2 ± 1.1[b]	−74.8 ± 2.3[b]	−91.8 ± 1.2[b]

[a] Values are the means ± SEM. Each number refers to 3–6 muscles of at least 20 surface fibers.

[b] $p < 0.01$ with respect to control.

However, using glycerol-treated sartorius muscles in which sodium conductance was blocked by tetrodotoxin (1×10^{-6} M), amantadine did not cause blockade of delayed rectification even after 1 hr of exposure (Fig. 4). The possibility that a rate-dependent block of potassium conductance would occur was checked by increasing the frequency of stimulation to develop delayed rectification from 1 Hz to 3 Hz. Even though the rate of activation was increased, amantadine had no significant effect on delayed rectification (Fig. 4).

Effects of amantadine on the amplitude and time course of the endplate current of the frog sartorius muscle. The effects of various concentrations of amantadine on the amplitude, rise time and half decay time of the epc, recorded at −90 mV, are presented in Table 5. Under normal conditions the epc had a rise time of 0.88 msec, a peak amplitude of 375 namps, and an exponential declining phase which had a half decay time of 1.6 msec. Amantadine (5×10^{-5} and 2×10^{-4} M) affected the amplitude, rise time and half decay time of the epc; the amplitude being the property most affected. For example, as shown in Fig. 5, exposure to the drug (2×10^{-4} M) for 60 min caused a reduction in the amplitude of the epc (recorded at −100 mV) by 80%, and shortened its rise time and half decay time by 15% and 30%, respectively. The onset of the action of amantadine (2×10^{-4} M) began after 30 min of incubation with the drug. Washing the preparation with normal Ringer's solution restored the preparations to control condition in about 120 min (Fig. 5C). Amantadine at 2×10^{-4} M did not affect the null potential of the epc since the value of the control null potential was −3.1 ± 1.6 mV, and in presence of amantadine after 2 hr exposure became −3.9 ± 1.3 mV. Semilog plots of the epc decay in control

TABLE 4

Effect of amantadine on the frog sartorius muscle action potential induced by indirect stimulation

Condition	Amplitude	Rate of rise	Time to half decay
	mV	*V/sec*	*msec*
Control	116 ± 3.1[a]	653 ± 19.3	0.52 ± 0.02
Amantadine (2×10^{-4} M)	92 ± 5.3[b]	350 ± 48.7[b]	1.46 ± 0.10[b]
Washing for 2 h	103 ± 1.4	581 ± 36.5	0.53 ± 0.03

[a] Values are the mean ± SEM. Each number refers to 4–8 single surface fibers of at least 3 muscles. The muscles were incubated for 60 min with the drug.

[b] $p < 0.01$ with respect to control.

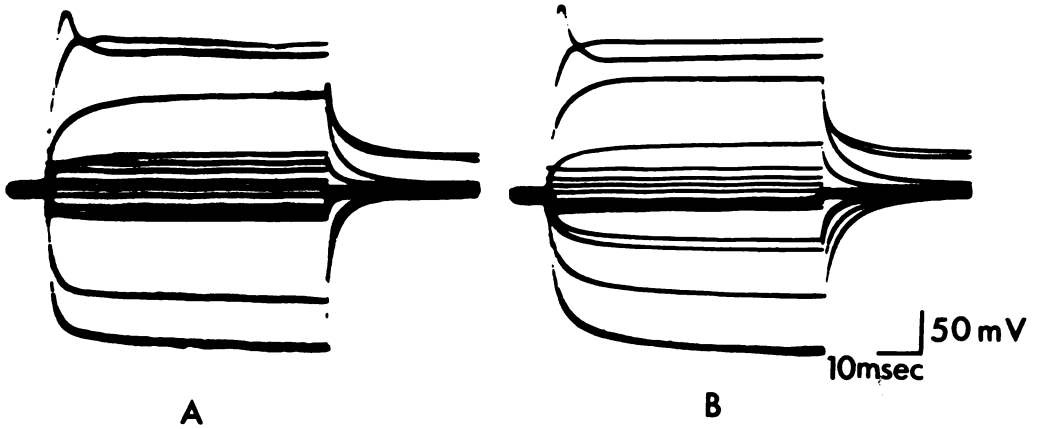

FIG. 4. *Electric potentials produced by anodal and cathodal pulses in glycerol shocked frog sartorius muscles*

Tetrodotoxin (3×10^{-6} M) is present at all times to block sodium conductance. A, shows responses in absence of, and B, after incubation of the muscle for 60 min with, amantadine (2×10^{-4} M). The resting membrane potential is −100 mV and −75 mV, respectively for A and B.

TABLE 5

Effect of amantadine on the amplitude and time course of endplate current in frog sartorius muscle recorded at −90 mV

Condition	Amplitude	Rise time	Half decay time
	nAmp	*msec*	*msec*
Control	375 ± 23 (74)[a]	0.88 ± 0.01	1.60 ± 0.02
Amantadine (5×10^{-5} M)	240 ± 19 (13)[c]	0.84 ± 0.03	1.42 ± 0.06[c]
Amantadine (2×10^{-4} M)	120 ± 19 (25)[c]	0.70 ± 0.03[c]	1.10 ± 0.06[c]
Washing[b]	353 ± 43 (8)	0.80 ± 0.03	1.59 ± 0.03

[a] The values refer to the mean ± SEM. The number in parenthesis refers to the number of endplates sampled in at least 4 muscles.

[b] Values refer only to muscles previously exposed to 2×10^{-4} M amantadine for 1 hr and then washing for 60–120 min.

[c] $p < 0.01$ with repsect to control.

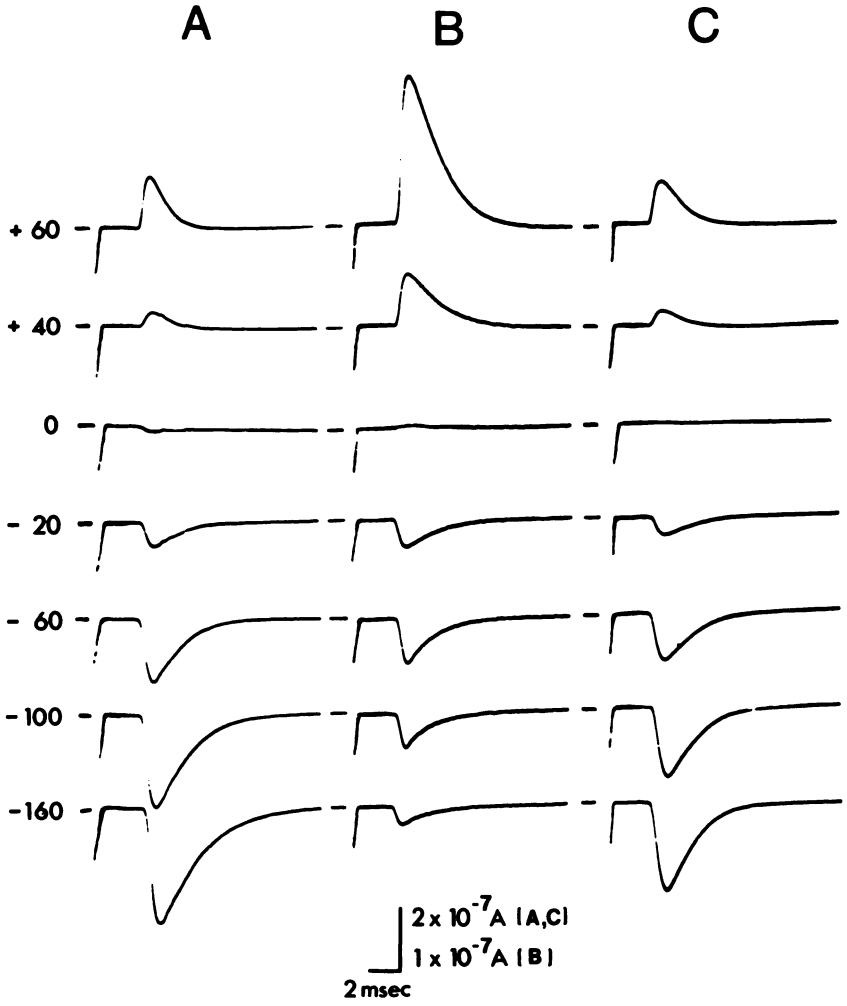

FIG. 5. *Series of epc's recorded at different membrane potentials in control (A), during exposure to amantadine (2×10^{-4} M) (B) and after 60 min washing (C)*

Each series is obtained from a single surface fiber of the frog sartorius muscle.

conditions and during exposure to amantadine (2×10^{-4} M) are shown in Fig. 6. Amantadine did not affect the exponential characteristics of the decay phase of the epc. A single exponential decay was observed under control conditions as well as at all concentrations of amantadine tested. On the spontaneous mepc, amantadine displayed an effect similar to that observed on the epc, i.e., the drug shortened the half decay time and the amplitude of the mepc.

Effects of amantadine on the endplate currents recorded at various membrane potentials. The effects of amantadine (2×10^{-4} M) on the epc's recorded at various membrane potentials are shown in Fig. 7. The drug induced negligible changes in linearity of the current-voltage relationship at positive potentials. However, as the membrane potential was made more negative, the slope conductance approached zero,

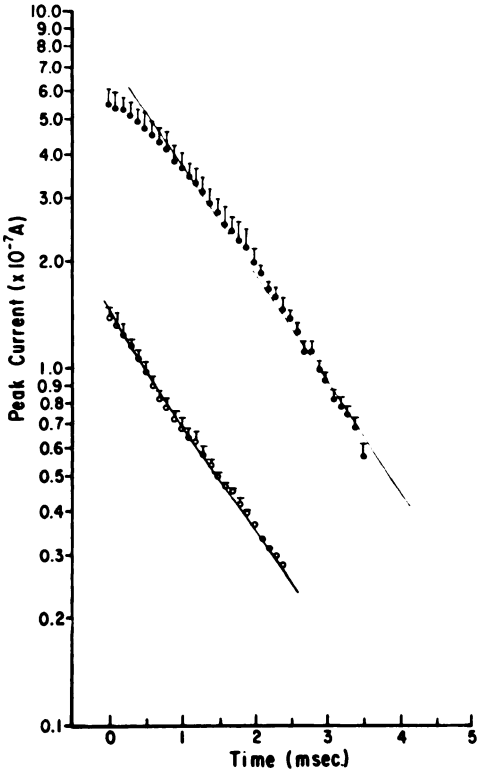

FIG. 6. *Semilogarithmic plot showing the exponential phase of decay of epc holding at −100 mV*

Control (●), in the presence of amantadine (2×10^{-4} M) (○). Each point is the mean of 15 fibers in four frog sartorius muscles ± SEM.

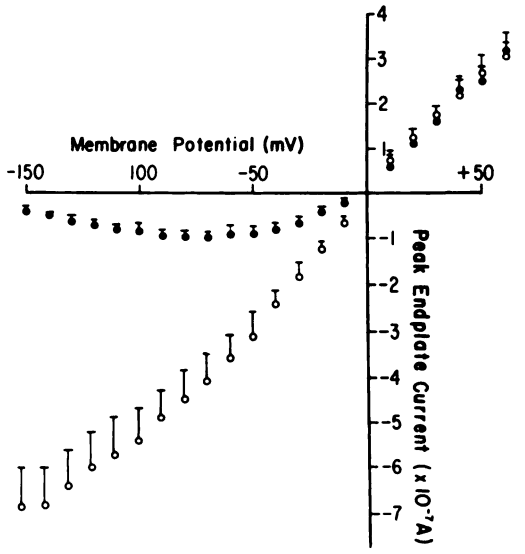

FIG. 7. *The relation between peak amplitude of the epc and membrane potential in control* (○) *and and in the presence of amantadine* (2×10^{-4} *M)* (●)

Each symbol represents the mena ± SEM of 10 fibers from four frog sartorius muscles.

and at approximately −70 mV it reversed its sign. At a potential of −150 mV the epc became even smaller than at −50 mV, giving the current voltage plot of the epc a bell shape at potentials from 0 to −150 mV. Thus, amantadine caused a significant departure from linearity of the epc amplitude-membrane potential relationship in the frog sartorius muscle. A similar pattern of drug activity was found on epc's recorded from the diaphragm muscles of the rat.

In the presence of amantadine the epc decay reversed sign as a function of voltage. Fig. 8 illustrates the amantadine-induced alteration in the relationship between half decay time and membrane potential. It should be mentioned that prior to addition of the drug the log plot of the half decay time varied in a linear fashion with membrane potential having a slope of -3.0 ± 0.09 V^{-1}. Amantadine markedly reduced the voltage sensitivity of the falling phase of the epc. Thus, during exposure to the drug, the relationship between half decay time and membrane potential, though linear, underwent a significant reduction in slope with subsequent reversal of slope to a value of 1.0 ± 0.09 V^{-1}. These effects of amantadine were reversible upon washing

the preparation for 90–120 min with Ringer's solution.

Effect of temperature on the action of amantadine. Studies on the effect of temperature may be helpful in differentiating the action of a given drug on the rate of diffusion of ACh out of the junctional cleft from those involving conformational changes of the macromolecules (4, 40). The effect of amantadine (2×10^{-4} M) at various temperatures on the half decay times of the epc is shown in Fig. 9. At the temperatures studied, amantadine significantly reduced the slope of the half decay time. Similarly, the peak amplitude of the epc was markedly reduced by the drug.

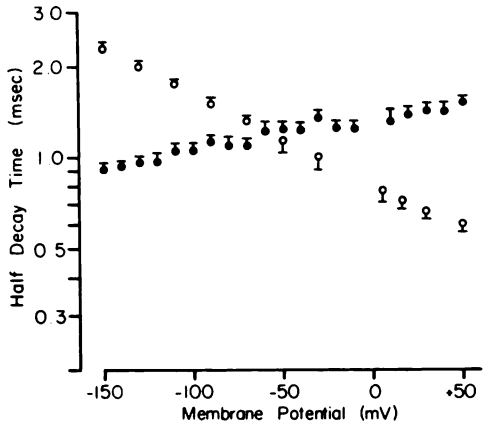

FIG. 8. *The relation between the half decay time of the epc and membrane potential in control* (○) *and in the presence of amantadine* (●) *(2×10^{-4} M)*

Each symbol represents the mean ± SEM of 20 fibers from five sartorius muscles.

The half decay times of the epc in control conditions and in the presence of amantadine (2×10^{-4} M) conform to the Arrhenius equations at temperatures varying from 10 to 30°C. The activation energies thus calculated at membrane potential of −90 mV were 11.5 KCal mole^{-1} for control and 8.2 KCal mole^{-1} for amantadine. These findings suggest that under control condition and in the presence of amantadine the epc decay follows first order reaction and also that an equivalent energy of activation regulates this process. Thus, alterations in temperature do not appear to affect the sites of action of amantadine.

Effects of amantadine on receptor-in-

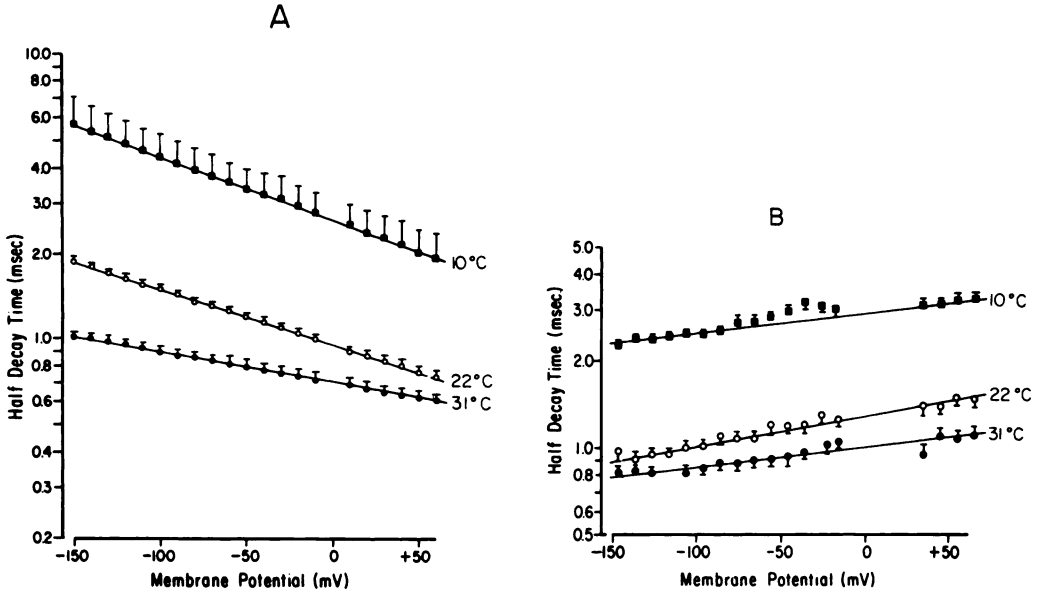

FIG. 9. *Semilogarithmic plot of the epc decay as a function of membrane potential recorded at various temperatures in control (A) and after exposure to amantadine (B) (2×10^{-4} M) for 30–60 min.*

Temperature is indicated with each curve. The slope for control at 10°C, 22°C and 31°C is −2.30 ± 0.19, −1.66 ± 0.09 and −1.15 ± 0.11 V^{-1}. For amantadine the slope is at +0.64 ± 0.06 (10°C), +0.96 ± 0.09 (22°C) and +0.52 + 0.08 (31°C) V^{-1}. Each point reflects the mean ± SEM of 8–16 endplates from five frog sartorius muscles.

duced cation fluxes and the binding of receptor and ionic channel ligands. If amantadine inhibits neuromuscular transmission, it should inhibit the specific ^{22}Na efflux from electric organ microsacs that is induced by activation of the ACh receptor. Indeed, amantadine (10^{-4} M) inhibited significantly the specific carbamylcholine-induced ^{22}Na efflux (Fig. 10). Higher inhibition was obtained by larger concentrations of amantadine. Inhibition was also obtained by the ionic channel drug H_{12}-HTX as well as by the ACh receptor inhibitor *d*-tubocurarine. To identify which of these two proteins was the target for amantadine, we studied the effects of amantadine on the binding of [^{3}H]ACh (1 μM) and [^{125}I]α-bungarotoxin (12.5 nM) (as indices of ACh receptor) and of [^{3}H]H_{12}-HTX (40 nM) (as an index of the postsynaptic ionic channel). The electric organ of *Torpedo* was selected for the *in vitro* study of the effect of drugs on these two kinds of binding, because this tissue is the richest known source for nicotinic ACh receptor (41) and presumably also its ionic channel. As previously shown [^{3}H]ACh binding was abolished by preincubation with 1 μM α-bungarotoxin and blocked to varying degrees by the ACh receptor agonist carbamylcholine and antagonist *d*-tubocurarine (Table 6). Binding of [^{125}I]α-bungarotoxin to the ACh receptor was also inhibited by *d*-tubocurarine and carbamylcholine. These drugs and toxin had no effect on [^{3}H]H_{12}-HTX binding except for little blockade by carbamylcholine. On the other hand, HTX, H_{12}-HTX and octahydro-HTX (H_2-HTX) blocked [^{3}H]H_{12}-HTX binding. By comparison with these drugs and toxins, amantadine acted like the histrionicotoxins by blocking [^{3}H]H_{12}-HTX binding at concentrations that did not affect [^{3}H]ACh binding to its receptor (Table 6). To obtain an inhibition constant (K_i) for amantadine, the effect of various concentrations of amantadine on the binding of [^{3}H]H_{12}-HTX (at 40 nM and 80 nM) was determined. The K_i was calculated from the Dixon plot (Fig. 11) to be 60 μM. At 100 μM amantadine had no effect on [^{3}H]ACh binding to its receptor (Table

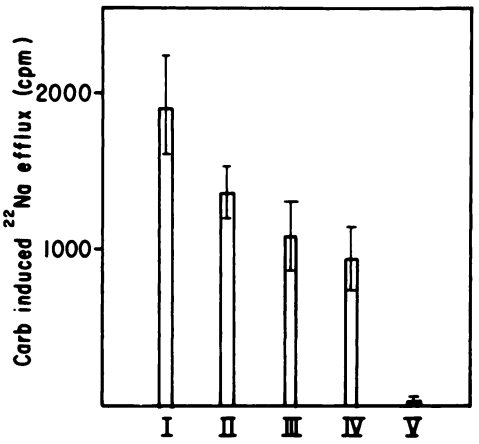

FIG. 10. *Histogram of the effect of drugs and toxins on the carbamylcholin-induced ^{22}Na efflux from Torpedo microsacs*

No drugs or toxins are added to control (V). Drug or toxin is added 36 min after start of ^{22}Na efflux, and carbamylcholine (10^{-4} M) is added at 39 min. Carbamylcholine alone (I); amantadine (10^{-4} M) + carbamylcholine (II); perhydrohistrionicotoxin (2×10^{-5} M) + carbamylcholine (III); perhydrohistrionicotoxin (2×10^{-5} M) + carbamylcholine (III) and *d*-tubocurarine (10^{-4} M) + carbamylcholine (IV). Bars represent ± standard deviation for 3 experiments.

TABLE 6

Blockade of binding of [^{3}H]acetylcholine and [^{3}H]perhydrohistrionicotoxin to ACh-receptor enriched membranes from the electric organ of Torpedo ocellata measured by equilibrium dialysis

Drug or toxin	[^{3}H]Perhydrohistrionicotoxin (40 nM)		[^{3}H]Acetylcholine (1 μM)	
	Conc	Binding as % of control	Conc	Binding as % of control
	μM		μM	
Carbamylcholine	100	86 ± 8	10	10 ± 3
d-Tubocurarine	100	103 ± 4	10	41 ± 5
α-Bungarotoxin	1	126 ± 7	1	1 ± 2
H_8-HTX	1	38 ± 2	1	99 ± 6
Amantadine	10	72 ± 2	10	108 ± 7
Amantadine	100	46 ± 1	100	100 ± 2

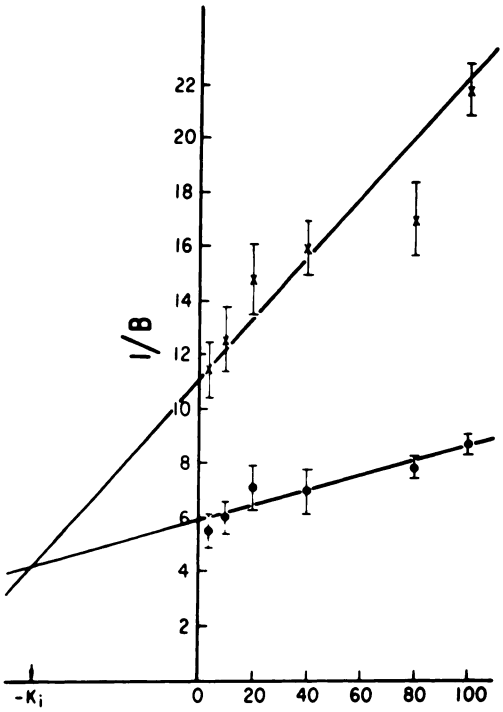

FIG. 11. *Dixon plot of the binding of [^{3}H]H$_{12}$-HTX to Torpedo microsacs in presence of varying concentrations of amantadine*

The [^{3}H]H$_{12}$-HTX concentrations used are 80 nM (●) and 40 nM (×). The abscissa represents concentration of [^{3}H]H$_{12}$-HTX in nM. Bars represent ± standard deviation for 3 experiments.

6), nor did it interfere with the binding of [^{125}I]α-bungarotoxin to the ACh receptor (Fig. 12).

Effect of amantadine on ACh esterase activity. ACh esterase activity in *Torpedo* microsacs was assayed by the Ellman *et al.* (37) method using acetylthiocholine from 50 to 1,000 μM and amantadine at 2 mM. The K_i value for amantadine was 7×10^{-4} M.

DISCUSSION

Three different experimental approaches used in the present research strongly suggest that amantadine interacts with the ionic channel of the ACh receptor. These are the electrophysiological determinations, the carbamylcholine-induced ^{22}Na efflux and the binding of [^{3}H]ACh and [^{125}I] α-bungarotoxin to the ACh receptor and [^{3}H]H$_{12}$-HTX to the ionic channel. Although mammalian and amphibian nerve-muscle preparations are used in the electrophysiological studies because of the amount of data available on these preparations, the *Torpedo* electric organ membranes are used in the biochemical studies because of the richness of this tissue in ACh receptor and its ionic channel. The synapses in these muscles and electric organ have similar pharmacological properties (41, 42).

Amantadine has strong postsynaptic action on frog (Table 1) and rat skeletal muscles related directly to a partial membrane depolarization (Table 3), followed by a prolongation of the falling phase of the action potential (Fig. 3) and development of spontaneous muscle contracture. But its most important effect is on the current generated by the reaction of the transmitter with the ACh receptor at the endplate region (Figs. 5–8 and Table 5).

The membrane depolarization induced by amantadine, at a concentration that causes marked effects on epc and action potential, is of the order of 20 mV in either rat or frog muscles (Table 3). Apparently, membrane depolarization is not related to an increase in Na^+ conductance since tetrodotoxin (3×10^{-6} M), or Na^+-free Ringer's solution, does not prevent or reverse the depolarization. Thus, this apparent lack of amantadine action on Na^+ and

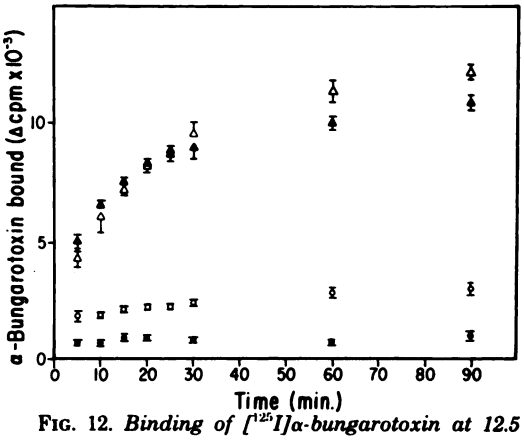

FIG. 12. *Binding of [^{125}I]α-bungarotoxin at 12.5 nM to Torpedo microsacs measured by filtration on Whatman GF/C discs*

[^{125}I]α-Bungarotoxin alone (Δ) and in presence of 10^{-4} M amantadine (▲), 10^{-4} M *d*-tubocurarine (○) or 10^{-6} M α-neurotoxin of *Naja n. siamensis* venom (●). Bars represent ± standard deviation for 3 experiments.

K^+ conductances is quite different from that obtained with drugs which increase Na^+ conductance such as batrachotoxin, grayanotoxin or veratridine (43–45). Because of the drug-induced depolarization, and the fact that delayed rectification is not sensitive enough to account for subtler changes in K^+ conductance, one has to assume that the prolongation of action potential may still be related to a partial block of K^+ conductance. However, recent experiments in our laboratories disclosed that a derivative of amantadine, rimantadine, caused neither membrane depolarization nor any significant effect on K^+ conductance (Albuquerque, unpublished). Yet the prolongation of the action potential by rimantadine still occurred. The prolongation of the action potential produced by amantadine (Fig. 3, Table 4) may also be related to the ability of the drug to block Na^+ inactivation in a way similar to that described by Rojas and Armstrong for pronase (46). If the effect of amantadine on the action potential is caused by blocking Na^+ inactivation, similar to the action of pronase, it is possible that this action of the drug is mostly on the inside of the membrane. Further experiments are necessary to identify the mechanism by which amantadine prolongs the action potential.

The potentiation of muscle twitch in the early phase of amantadine action may be explained by a number of factors such as the change in the threshold for depolarization, the ability of the drug to cause a decrease in membrane potential and the prolongation of action potential. Although amantadine produces this marked effect on the muscle membrane, it does not cause apparent depolarization of the nerve terminal. No increase in spontaneous transmitter release is detected (Table 1).

The effect of amantadine on the epc discloses a voltage-dependent attenuation of peak amplitude (Figs. 6, 7) and thus a marked departure from linearity in the current-voltage relationship. In contrast to the peak amplitude observed in control epc's, which responds linearly to changes in the driving force between +60 and −100 mV, and exhibits a small curvature at −100 to −160 mV (3, 4), amantadine at 2×10^{-4} M completely changes the current-voltage relationship except at positive potentials (see Fig. 7). This lack of effect of amantadine on epc's at positive membrane potentials strongly suggests that the drug is not an antagonist of the ACh receptor like *d*-tubocurarine and α-bungarotoxin (6, 47). Furthermore, in the presence of amantadine, the slope of the half decay time-membrane potential relationship undergoes reversal, such that the epc now becomes faster with hyperpolarization (Fig. 8). Although a small shift in the slope of the half decay time-membrane potential relationship has been reported for atropine (2, 48) and the local anesthetics (e.g., QX314) (49), these were not as pronounced as that seen with amantadine. Indeed, during exposure to amantadine (2×10^{-4} M), the slope of the logarithm of the half decay time is $+1.0\ V^{-1}$, while in control it is $-3.0\ V^{-1}$. The effect of amantadine on half decay time was observed at negative as well as positive potentials, whereas the effect on peak amplitude was observed only at negative potentials. Since amantadine does not change the null potential, it is suggested that the drug does not affect selectively any ionic species. This is in agreement with the hypothesis that there is only one type of channel for both K^+ and Na^+ at the cholinergic receptor-channel complex (50–52). Amantadine, like HTX (7, 9, 12, 30) and tetraethylammonium (53), reacts with the endplate ionic channel in resting and active states. Thus, to explain the effect of amantadine on the epc, the drug must affect the ionic channel in at least two distinct manners: 1) amantadine may react with the receptor-channel complex in the closed conformation, thus producing a marked departure in linearity in the current voltage relationship, and 2) the drug reacts with the receptor-channel complex in open conformation causing in this way the marked changes observed in the relationship between half decay time and membrane potential. It is to be noted that although amantadine inhibits ACh-esterase, yet its K_i (7×10^{-4} M) is much higher than the concentrations used to affect epc's.

The lengthening of the half-decay time at positive membrane potentials induced

by amantadine suggests that the drug remains bound to its site at positive potential and generates a large population of open channels, thus explaining the slope of 1.0 V^{-1} seen in the presence of the drug. Further studies are necessary to evaluate this novel effect of amantadine at positive potentials.

Similarly to HTX, amantadine does not react with the recognition site of ACh, but rather with the ionic channel of the ACh receptor. Indeed, the results of binding studies are in full agreement with this conclusion. In a previous study (10) we provided evidence to suggest that binding of [^{3}H]H_{12}-HTX to *Torpedo* electric organ membrane (K_d of 4×10^{-7} M) was to the ionic channel of the ACh receptor. This binding has its own drug sensitivity, being inhibited best with HTX analogues, and less so by several local anesthetics. Evidently, amantadine, like HTX, inhibits [^{3}H]H_{12}-HTX binding competitively (Fig. 11) but at the same concentration does not inhibit binding of [^{3}H]ACh (Table 6) or [^{125}I]α-bungarotoxin (Fig. 12) to the ACh receptor. The effectiveness of amantadine in binding to the ionic channel is intermediate between HTX and local anesthetics with a K_i for inhibition of [^{3}H]H_{12}-HTX of 6×10^{-5} M. On the other hand, as expected, both receptor antagonists (e.g., *d*-tubocurarine) and ionic channel antagonists (e.g., HTX) inhibit the ^{22}Na efflux from electric organ microsacs that is induced by binding of carbamylcholine to the ACh receptor, as does amantadine (Fig. 10).

In conclusion, amantadine blocks neuromuscular transmission of mammalian and amphibian muscles in a voltage dependent manner; these effects being related to a direct reaction with the ionic channel of the ACh receptor. The data support the notion that amantadine reacts with the ionic channel in either resting or active conformations and has no effect on the ACh receptor at the concentrations used.

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