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Structure-Activity Relationships of Amantadine

I. Interaction of the *N*-Alkyl Analogues with the Ionic Channels of the Nicotinic Acetylcholine Receptor and Electrically Excitable Membrane

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SUMMARY

In this study the effects of amantadine (1-adamantanamine) and its *N*-alkyl-substituted analogues [*N*-methyl- (NMA), *N*-ethyl- (NEA), *N*-propyl- (NPA), *N*-butyl- (NBA), and *N,N*-diethyl-amantidine (NNDEA)] were investigated on ionic channels of the electrically excitable membrane and of the nicotinic acetylcholine (ACh) receptors in frog sartorius muscles and on the binding of perhydrohistrionicotoxin (H_{12}-HTX) to isolated membranes of the electric organ of the electric ray *Torpedo*. Amantadine and each analogue blocked the indirectly elicited twitch, but NPA, NBA, and NNDEA also potentiated the directly elicited twitch. The order of potency in inhibiting the indirect twitch was: NEA = NPA = NNDEA (10 μM) > NMA (15 μM) > NBA (40 μM) ≫ amantadine (130 μM). Neither amantadine nor its *N*-alkyl analogues affected miniature end-plate potential frequency or resting membrane potential but decreased miniature end-plate potential amplitude. Each compound prolonged the directly elicited action potential but did not alter delayed rectification. All of the compounds induced a concentration-dependent depression of the peak end-plate current (EPC) amplitude at negative membrane potentials and induced nonlinearity in the response at membrane potentials more negative than −40 mV. The order of potency in inhibiting the EPC (at −90 mV) was NNDEA (<0.5 μM) > NPA (<1.0 μM) > NBA (<2.0 μM) > NEA (19 μM) > NMA (42 μM) > amantadine (64 μM). Only NPA, NBA, and NNDEA depressed the peak EPC amplitude at positive membrane potentials as well. The shortening of the time constant of EPC decay by all compounds was concentration-dependent. At the higher concentrations examined, the slope of the relationship between the time constant of decay and membrane potential was reversed for all compounds. Only NPA induced a double-exponential decay of the EPC at positive membrane potentials. Neither amantadine nor its *N*-alkyl analogues inhibited the binding of [^{3}H]ACh to its receptor in *Torpedo* electroplax but they inhibited the binding of [^{3}H]H_{12}-HTX binding to the ionic channel sites of the ACh receptor. The K_i for inhibition of [^{3}H]H_{12}-HTX binding was NEA = NNDEA (15 μM) > NMA (30 μM) > NPA = NBA (40 μM) > amantadine (60 μM). A gross correlation exists between their ability to block the indirect muscle twitch, miniature end-plate potential amplitude, peak EPC amplitude and the binding of [^{3}H]H_{12}-HTX. But, no correlation was found between these potencies and their antiviral activity. It is suggested that these compounds may interact with the ionic channel of the ACh receptor in its open and closed conformation.

INTRODUCTION

Several molecular probes have been used to elucidate the sequence of events which initiates activation of the nicotinic receptor followed by a voltage-dependent conformational activation of the ionic channel and culminating with the appearance of a transient EPC.[1] Although α-bungarotoxin has been employed as a specific agent for

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[1] The abbreviations used are: EPC, end-plate current; ACh, acetylcholine; RMP, resting membrane potential; EPP, end-plate potential; MEPP, miniature end-plate potential; H_{12}-HTX, perhydrohistrionicotoxin; NMA, *N*-methylamantadine; NEA, *N*-ethylamantadine; NPA, *N*-propylamantadine; NBA, *N*-butylamantadine; NNDEA, *N,N*-diethylamantadine; τ_{EPC}, time constant of EPC decay.

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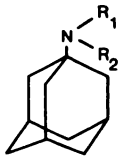
the detection of the nicotinic ACh receptor (1), unlabeled and radiolabeled histrionicotoxins and local anesthetics have been used as specific probes, first to establish the existence of sites on the ionic channel associated with the nicotinic ACh receptor and later to characterize its kinetics (2–7).

Although many compounds with a variety of chemical structures have been used, only two basic alterations have been observed in the kinetics of the EPC. One group, which includes atropine (ref. 8 and references therein), histrionicotoxin (9), and the quaternary lidocaine derivative QX-314 (10, 11), shortens the EPC rise time and increases its decay rate without affecting its single exponential nature. The second group, procaine (12, 13), the lidocaine derivative QX-222 (10, 11), and scopolamine (9), shortens the EPC rise time but converts the decay phase to a multiexponential function consisting of at least two distinct components, one more rapid and another slower than the decay of control EPCs.

Amantadine hydrochloride (1-adamantanamine hydrochloride), which is an antiviral agent effective against A2 (Asian) influenza in animals (14) and in man (15), is also effective in the treatment of human Parkinsonism (16). Although the apparent antiviral activity results from the ability of the drug to prevent viral penetration into the host cell, its antiparkinson action may depend on its capacity to increase the synthesis and release of dopamine from dopaminergic cells of the basal ganglia (17). However, it was reported that amantadine blocked neuromuscular transmission by reducing the response of muscle postjunctional membrane to ACh (18). This block was found to be due mostly to its interaction with ionic channel sites of the ACh receptor (19, 20). The availability of five *N*-alkyl-substituted analogues of amantadine (Fig. 1) offered the unique opportunity to study the mechanism of action of this class of agents and their ability to affect various conformational states of the ACh receptor/channel molecule. The objective of the study was therefore to compare the effects of amantadine and its analogues in their abilities to alter neuromuscular transmission via action on ionic channel sites and also to understand further the kinetic properties of the ACh receptor/channel complex based on the structure-activity relationships of these compounds.



Compound	Mol. Wt.	R_1	R_2
Amantadine	187.8	-H	-H
NMA	201.7	$-CH_3$	-H
NEA	215.8	$-C_2H_5$	-H
NPA	229.8	$-C_3H_7$	-H
NBA	243.8	$-C_4H_9$	-H
NNDEA	243.8	$-C_2H_5$	$-C_2H_5$

FIG. 1. *Structure and molecular weight of amantadine and its N-alkyl-substituted analogues*

METHODS

Electrophysiological Experiments

Animals and preparations. All experiments were performed at room temperature (20–22°) on sciatic-sartorius muscle preparations of the frog *Rana pipiens*. In voltage-clamp experiments, the muscles were treated with 400–600 mM glycerol contained in physiological solution for 60 min followed by a 60-min wash with normal physiological solution to uncouple excitation from contraction (21).

Solutions and Drugs. The normal physiological solution had the following composition (millimolar): NaCl, 115.5; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4, 1.3; and NaH_2PO_4, 0.7. The solution was bubbled with 100% O_2 and had a pH of 6.9–7.1. Drugs were stored as refrigerated stock solutions and were diluted with physiological solution immediately before use. The stock solutions were as follows: amantadine hydrochloride and its *N*-alkyl-substituted analogues (10^{-2} M) (E. I. DuPont and Company, Wilmington, Del.), *d*-tubocurarine chloride (ICN K & K Laboratories, Plainview, N. Y.) and tetrodotoxin (Sankyo Company, Ltd., Tokyo, Japan, via Calbiochem, San Deigo, Calif.; 3×10^{-4} M) in distilled water.

Recording details. For twitch studies, the sciatic nerve to the sartorius muscle was stimulated at 0.05 Hz with supramaximal pulses having a duration of 0.05 msec via an Ag-AgCl salt bridge electrode connected to a wet electrode. Direct stimulation of the muscle was accomplished by applying supramaximal rectangular pulses of 1.0- to 2.0-msec duration at 0.05 Hz through a bipolar platinum electrode placed around the middle portion of the muscle. The muscle tension generated by both direct and indirect stimulation (at a total of 0.1 Hz) was recorded with Grass FT.03 force displacement transducer attached to a Grass (Model 7) polygraph. At least 30 min were allowed for muscle stabilization in the chamber. For recording of intracellular potentials, muscles were stretched slightly beyond resting length and pinned to the paraffin lining of a Plexiglas plate having a plano-convex lens in the center and then placed in a 15-ml capacity bath. For end-plate and action potentials the sciatic nerve was stimulated by supramaximal pulses of 0.05-msec duration via bipolar platinum electrodes. Intracellular recordings of the RMP, action potential, EPP, MEPP, EPC, and delayed rectification from the surface fibers of the muscle were made in a conventional manner (20, 22, 23) with 3 M KCl-filled microelectrodes (2–7 Mohm). Muscle action potentials were recorded by inserting two microelectrodes into the same surface fiber 100–150 μm apart, one to pass a current and the other to record the action potential. In order to obtain more homogeneous responses, the RMP was preset to a value of about −90 mV by passing a constant anodal current across the membrane through the stimulating microelectrode.

The maximal rate of rise of action potential (dV/dt) was measured by means of an RC circuit (100 Kohm, 100 pF) and displayed on the second beam of an oscilloscope. The voltage-clamp circuit used was similar to that previously described (24). Voltage-clamp errors were less than 5% of the unclamped EPP. The time constant of the clamping circuit (10–90%) with 2- to 7-Mohm 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. Waveforms were sampled by a PDP 11/40 computer.

Biochemical Techniques

Membrane preparation. Membranes were prepared from the electric organ of *Torpedo ocellata* (obtained from Alexandria, Egypt, and stored at −90°) by homogenization (20% w/v) in an ice-cold solution of 90 mM KCl, 10 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4) and centrifugation of the supernatant of a 5,000 × *g* 10-min centrifugation for 60 min at 30,000 × *g*. This pellet was resuspended in Krebs' original Ringer-phosphate solution (millimolar): NaCl, 107; KCl, 4.8; $CaCl_2$, 0.65, $MgSO_4$, 1.2; and Na_2HPO_4, 15.7 (pH 7.4). The final protein concentration ranged from 1 to 2 mg/ml, and the maximal numbers of binding sites for [^{3}H]ACh and [^{3}H]H_{12}-HTX were 0.7 and 0.5 nmole/mg of protein, respectively.

Equilibrium dialysis. Equilibrium dialysis was used to study the binding of [^{3}H]ACh (49.5 mCi/mmole; New England Nuclear Corporation, Boston, Mass.) to the ACh receptor sites in the electric organ membranes as previously described (3). Membrane preparation (0.5 ml) in a dialysis bag was placed in Krebs' original Ringer-phosphate (25 ml) containing [^{3}H]ACh (in the absence or presence of drug) and shaken for 4 hr at 21°. Triplicate samples of 50 μl were then taken from each dialysis bag and bath, and the radioactivity was counted in toluene-based scintillation solution. The radioactivity in the bag in excess of that in the bath sample represented the amount of ligand bound to the membranes. In order to inhibit all cholinesterases without affecting the binding of ACh to its receptor, diisopropyl fluorophosphate was added to the membranes, at a final concentration of 1 mM, 1 hr before the start of dialysis, and was present at 100 μM during dialysis.

Centrifugal assay. Centrifugal assay was used to study the binding of [^{3}H]H_{12}-HTX (21 Ci/mmole) to the channel sites of the ACh receptor in electric organ membranes as previously described (3). Samples (10 μl) of different concentrations of [^{3}H]H_{12}-HTX in ethanol were added to 1-ml samples of the *Torpedo* membrane preparation, mixed, and incubated for 60 min at 21° in siliconized 1.5-ml polyethylene microcentrifuge tubes. The drug to be tested was added to the membranes 30 min (at 21°) prior to the addition of [^{3}H]H_{12}-HTX. The incubation mixture was then centrifuged at 30,000 × *g* for 60 min, and three samples (50 μl each) were taken from the mixture before, as well as from the supernatant after, centrifugation and the radioactivity was counted. Excess radioactivity in the former represented the bound [^{3}H]H_{12}-HTX. Specific binding to the ionic channel sites was that inhibited with 1 mM amantadine, which had been shown to inhibit competitively the specific [^{3}H]H_{12}-HTX binding to these sites (20).

Statistics. All values are expressed as the mean ± standard error of the mean. All p values ≤ 0.05 were taken to be statistically significant.

RESULTS

Effects on directly and indirectly elicited muscle contractions. The effect of amantadine and its *N*-alkyl-substituted analogues on twitch tension was compared on the frog sciatic nerve-sartorius preparation. Amantadine (500 μM) blocked the indirectly elicited twitch within 10 min without affecting the directly elicited twitch (Fig. 2). The onset of blockade by amantadine (500 μM) began within 0.3 ± 0.1 min and was nearly complete at 10 min (Table 1). There was never a contracture during exposure to amantadine, and complete recovery of the indirect twitch occurred after washing with physiological solution for 60 min. NMA and NEA had similar effects on the sartorius muscle in that they blocked the indirectly elicited twitch but not the directly elicited twitch. But NPA, NBA, and NNDEA potentiated the direct twitch in addition to blocking the indirectly elicited twitch. Typical recordings of the effect of NMA and NPA on twitch tensions are shown in Fig. 2. The effects of varying concentrations of amantadine and its *N*-alkyl-substituted analogues on the direct and indirect twitches are summarized in Table 1. Maximal potentiation of direct twitch with NPA, NBA, and NNDEA occurred within 20 min and to a similar extent at 100 μM (Table 1). The log dose-effect relationship for amantadine and its *N*-alkyl analogues is shown in Fig. 3 and reveals essentially three groups: (a) amantadine; (b) NBA; and (c) NMA, NEA, NPA, and NNDEA. The 50% inhibitory concentration (IC_{50}) for blockade of the indirect twitch with amantadine was 130 μM; with NMA, 15 μM; with NEA, NPA, and NNDEA, 10 μM; and with NBA, 40 μM. Thus, the ana-

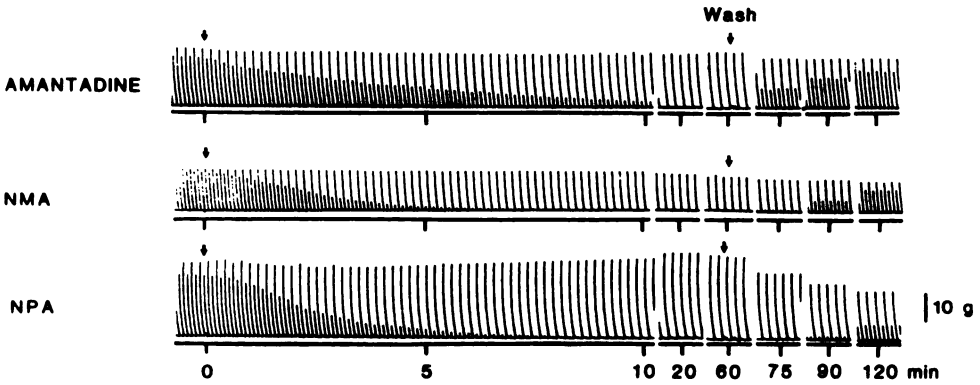


FIG. 2. *Typical response of frog sartorius muscle to amantadine, NMA, and NBA*

Each of the tracings was obtained from polygraph records of muscle twitch during alternating supramaximal direct and indirect stimulation (0.1 Hz each). The drugs were each added at the *arrow* (zero time) and washed at about 60 min. In each case the indirect response is blocked; with amantadine (500 μM) and NMA (100 μM), the direct response is unaffected up to 60 min, but it is potentiated by NPA (100 μM). No contractures were observed, but there is an indication in the falling phase of the twitch during exposure to NPA that the twitch is prolonged.

TABLE 1

Effect of amantadine and analogues on directly and indirectly elicited twitches of the frog sciatic-sartorius muscle preparation

Values shown are the means ± standard deviation of at least three muscles that were stimulated continuously for 30 min prior to exposure to the compound at room temperature (21°) with no contracture occurring at any concentration

Drug	Concentration	Indirect twitch			Direct twitch	
		Onset	Time to maximal block	Block at 60 min	Time to maximal potentiation	Potentiation
	μM	*min*	*min*	%	*min*	%
Amantadine	50	2.3 ± 0.5	12.2 ± 1.5	18.5 ± 3.0	None	None
	100	1.0 ± 0.2	11.6 ± 4.2	28.6 ± 5.0		
	200	0.5 ± 0.2	11.6 ± 5.7	86.3 ± 1.8		
	500	0.3 ± 0.1	10.0 ± 5.0	99.5 ± 2.0		
NMA	10	5.0 ± 1.0	20.0 ± 2.5	22.5 ± 3.0	None	None
	20	2.0 ± 0.5	15.0 ± 3.5	73.0 ± 5.5		
	50	0.5 ± 0.3	10.0 ± 1.7	85.0 ± 8.0		
	100	0.2 ± 0.1	5.1 ± 0.8	98.7 ± 1.3		
NEA	5	10.0 ± 6.0	30.8 ± 7.8	5.0 ± 1.0	None	None
	20	1.0 ± 0.4	20.0 ± 4.7	63.0 ± 4.4		
	50	0.8 ± 0.2	15.0 ± 6.6	76.3 ± 7.7		
	100	0.5 ± 0.2	12.0 ± 4.5	100.0 ± 0.0		
NPA	5	20.0 ± 4.0	60.0 ± 18.0	5.0 ± 3.0		
	10	5.0 ± 2.0	30.0 ± 10.0	24.0 ± 5.0		
	20	3.0 ± 1.5	30.0 ± 8.0	63.0 ± 9.0		
	100	0.5 ± 0.4	10.0 ± 5.5	100.0 ± 0.0	21.0 ± 7.0	145.0 ± 26.0
NBA	50	1.1 ± 0.2	15.1 ± 2.3	59.1 ± 12.0		
	100	0.5 ± 0.1	15.0 ± 4.0	90.0 ± 2.0	20.5 ± 4.0	133.0 ± 12.0
NNDEA	5	10.0 ± 2.0	30.0 ± 5.0	12.0 ± 4.0		
	10	1.0 ± 0.5	20.0 ± 4.0	33.0 ± 7.0		
	50	1.0 ± 0.2	17.5 ± 3.0	92.0 ± 7.5		
	100	0.5 ± 2.1	10.0 ± 2.0	96.7 ± 3.3	20.0 ± 0.5	143.0 ± 17.0
	500	0.2 ± 0.1	5.0 ± 1.5	100.0 ± 0.0	10.0 ± 3.0	143.0 ± 16.0

logues were more potent than the parent compound amantadine.

Effects on the resting membrane potential and on the spontaneous transmitter release of the sartorius muscle of the frog. Both amantadine and its substituted analogues decreased the amplitude of the MEPPs in a concentration-dependent fashion (Table 2). The membrane potential of the muscle fibers under control conditions was −92.2 ± 0.5 mV (36 muscles/165 fibers). Except for an 8% decrease in the membrane potential during exposure to amantadine (100–200 μM), none of the analogues caused any depolarization. The extent of the decrease in spontaneous MEPP amplitude was dependent upon the particular analogue used and also to some extent on the time of exposure. Amantadine (100 μM) decreased the MEPP amplitude by 45% at 15 min and by 74% at 30 min (Table 2) with a corresponding, but apparent, decrease in MEPP frequency of about 45–47% at 15 min and 30 min. At 200 μM, amantadine blocked the MEPPs completely within 5 min. Like amantadine, 20 μM NMA reduced the MEPP amplitude by 76% (Table 2) and MEPP frequency by 14% at 30 min, indicating that NMA was more potent than amantadine in this respect as well as in blocking the indirectly evoked twitch (Fig. 3). Increasing the concentration of NMA to 50 μM completely blocked MEPPs. Similarly greater potency of NPA and NBA was observed (Table 2). As the concentration of each of the analogues was increased, there was a decrease in the amplitude of MEPPs and an apparent decrease in MEPP frequency. Neither amantadine (20) nor any of its analogues depolarized the presynaptic nerve terminal, as suggested by their ineffectiveness in causing an increase in MEPP frequency and quantal content.

Effects on the action potential-generating mechanism. Figure 4 shows the effect of amantadine and three representative analogues (NMA, NPA, and NNDEA) on the directly elicited action potential in surface fibers of the frog sartorius muscle. Both amantadine and its analogues prolonged the falling phase of the action potential, but there was no effect on the action potential threshold, overshoot, amplitude, or rate of rise. The half-decay time of the control action potential was 0.80 ± 0.03 msec (30 fibers). Amantadine (200 μM), NMA (25 μM), NPA (25 μM), NBA (100 μM), and NNDEA (100 μM) increased the half-decay time to 188%, 150%, 150%, and 163% of control, respectively. The prolongation of action potential duration caused by amantadine or its analogues suggests that they may have an effect on potassium conductance. However, neither amantadine nor any of its analogues blocked delayed rectification in tetrodotoxin-treated sartorius muscle even after 60 min of exposure to the ana-

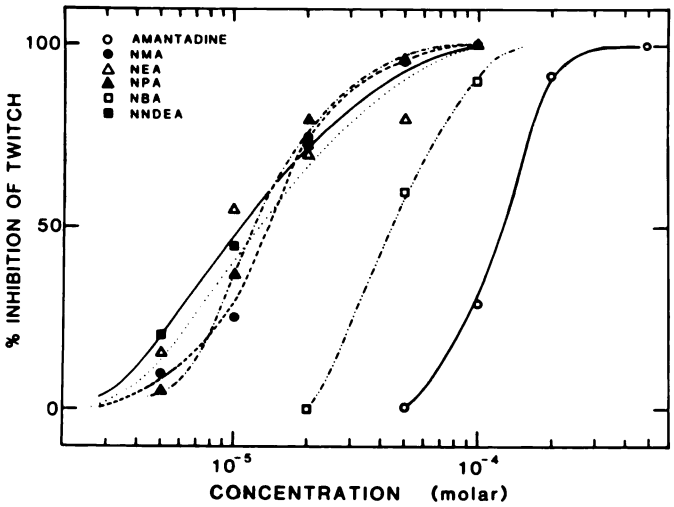


FIG. 3. *Log-dose relationship of the effect of amantadine and analogues on the indirectly elicited twitch of frog sartorius muscles*

Each point represents the mean of results with three to five individual muscles, and each curve was constructed from four to five concentrations. Each muscle was exposed to one drug for 1 hr, at which time twitch amplitude was measured. The muscles were stimulated alternately directly and indirectly (0.1 Hz each). Standard errors are not shown but were less than 5% of the means.

logues. The possiblity that a rate-dependent block of potassium conductance would occur was examined by increasing the frequency of stimulation. Even though the rate of stimulation was increased to 5 Hz, neither amantadine nor any of its analogues had significant effect on delayed rectification (data not shown).

Effects on the amplitude and time course of the EPC of the frog sartorius muscle at −90 mV. In light of results obtained with amantadine and the differential effects of the analogues on twitch, it was of interest to determine their effects on the EPC amplitude and time course. Amantadine affected both the peak EPC amplitude and time constant of EPC decay (τ_{EPC}) recorded at −90 mV. The normal EPC had a mean rise time of 0.82 msec, a peak amplitude of 0.38 μamp, and an exponential declining phase with a τ_{EPC} of 2.07 msec. These values are in close agreement with ones obtained for control EPC reported by others (8, 20, 25). Table 3 shows the alterations of EPC during exposure to amantadine or any of its analogues. When exposed to amantadine (100 μM) for 30–60 min, the peak EPC amplitude was reduced by 75% and τ_{EPC} was shortened by 42%, all changes being highly significant ($p < 0.01$). Amantadine had no effect on the rise time of the EPC nor did it change the equilibrium or null potential of the EPC. A single exponential decay was observed under control conditions as well as at all concentrations of amantadine used. These values for amantadine are also in agreement with those previously reported (20).

Typical traces under control conditions, and in the presence of amantadine (100 μM), NMA (20 μM), and NPA (50 μM) are illustrated in Fig. 5. Like amantadine, all of the analogues depressed the EPC amplitude as well as shortened the τ_{EPC}. The reductions of EPC amplitude in the presence of NMA (50 μM), NEA (50 μM), NPA (50 μM), NBA (100 μM), and NNDEA (50 μM) were 74%, 75%, 84%, 84%, and 86% and the corresponding shortenings of τ_{EPC} were 60%, 60%, 75%, 77%, and 54%, respectively. None of these analogues altered the null potential of the EPC. The results shown in Table 3 for the peak EPC and in Table 4 for the approximate concentration of each compound which reduces the peak EPC (at −90 mV) by

TABLE 2

Effect of amantadine and analogues on spontaneous miniature end-plate potential frequency and amplitude in frog sartorius muscles

The frequency and amplitude were evaluated in 5–12 fibers in each of 3–7 muscles at each concentration with 100–200 single potentials measured per muscle fiber. All values are means ± standard error of the mean.

Drug	Concentration	Miniature end-plate potential amplitude			
		0 Min	15 Min	30 Min	60 Min
	μM		mV		
Amantadine	0	0.42 ± 0.09			
	100		0.23 ± 0.01	0.11 ± 0.01	0.05 ± 0.01
	200		0	0	0
NMA	0	0.55 ± 0.07			
	5		0.50 ± 0.03	0.46 ± 0.01	0.44 ± 0.02
	10		0.30 ± 0.02	0.25 ± 0.01	0.22 ± 0.02
	20		0.14 ± 0.01	0.13 ± 0.01	0.11 ± 0.01
	50		0.01 ± 0.00	0	0
	100		0	0	0
NPA	0	0.38 ± 0.01			
	2		0.35 ± 0.01	0.33 ± 0.01	0.31 ± 0.01
	8		0.25 ± 0.01	0.23 ± 0.01	0.20 ± 0.00
	50		0.11 ± 0.01	0.08 ± 0.01	0.08 ± 0.02
	100		0	0	0
NBA	0	0.38 ± 0.02			
	100		0.22 ± 0.02	0.10 ± 0.01	0.05 ± 0.01
	200		0.14 ± 0.01	0.11 ± 0.01	0.05 ± 0.00
	500		0	0	0

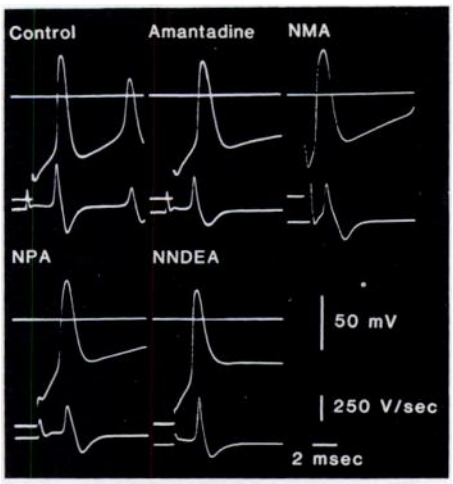


FIG. 4. *Directly elicited action potentials in surface fibers of the frog sartorius muscle in the presence of amantadine, NMA, NPA, and NNDEA*

Muscles were exposed to each drug for 30 min before recordings were begun, and all records were obtained between 30 and 60 min after exposure. The *upper trace* shows the action potential and the *lower trace* shows its first derivative (dV/dt); the *horizontal line* in each tracing represents the zero potential. The membrane was preset at −90 mV by anodal hyperpolarization (see Methods) prior to generating the action potential. Amantadine, 100 μM; NMA, 25 μM; NPA, 100 μM; NNDEA, 100 μM.

50% (i.e., IC_{50}) reveal that the analogues were more potent than amantadine in reducing the peak EPC amplitude as well as in shortening τ_{EPC}. The effects became more pronounced with the increase in length of the R_1 chain and, for example, 50 μM NPA caused more shortening of τ_{EPC} (75%) than did 100 μM amantadine (42%) or 50 μM NMA (60%) (Table 3).

Effects on EPCs recorded at various membrane potentials. A more detailed study of the induced alteration of the EPC requires clamping the end-plate membrane over a wide range of potentials. The peak amplitude of the normal EPC responded linearly to changes in the driving force at potentials between −150 and +60 mV (Figs. 5–9). A small deviation from linearity was frequently observed as the membrane was clamped at potentials more negative than −130 mV. The presence of nonlinearity in the current-voltage relationship at higher negative potentials was previously reported (8, 25, 27, 28). Amantadine, at a concentration of 200 μM or greater, effectively suppressed the tendency of the EPC amplitude to follow the driving force at potentials more negative than −40 mV; as the membrane was hyperpolarized further, the amplitude of the EPC declined. However, the relationship between peak EPC amplitude and membrane potential remained linear between −30 and +60 mV (Fig. 6). A similar type of effect on peak EPC amplitude at negative as well as positive membrane potentials has been observed with NMA (Fig. 7) and NEA. Neither amantadine, NMA, nor NEA had any depressant effect on peak EPC amplitude at positive membrane potentials. NPA (Fig. 8) and NBA (Fig. 9) as well as NNDEA (data not shown) reduced the peak EPC amplitude at both negative and positive membrane potentials, and the effect was dose-dependent (Figs. 8 and 9).

In addition to changes in peak EPC amplitude, τ_{EPC} also varied with changes in the membrane electric field, as observed by Takeuchi and Takeuchi (29). In control experiments, the EPC decayed progressively faster in a log-linear fashion as the membrane was depolarized from −150 to +60 mV, having a slope of $-2.5 + 0.01\ V^{-1}$ (Figs. 6–9B). Amantadine markedly reduced the voltage sensitivity of the falling phase of the EPC (Fig. 6). During the exposure to amantadine, the slope of the relationship between τ_{EPC} and membrane potential was significantly reduced at membrane potentials between −50 and −150 mV, but increased at potentials of +20 to +50 mV. Thus, the slope of the relationship was reversed with a value of

TABLE 3

Effect of amantadine and analogues on the amplitude and time course of end-plate currents in the frog sartorius muscle recorded at −90 mV

Values in parentheses refer to the number of single fibers sampled in at least three muscles.

Drug	Concentration	Amplitude	Rise time	Half-decay time
	μM	× 10^{-7} amp	msec	msec
Control	100	3.79 ± 0.21 (77)	0.82 ± 0.02	2.07 ± 0.25
Amantadine	100	0.95 ± 0.04 (7)[a]	0.70 ± 0.06	1.20 ± 0.30[a]
NMA	10	3.50 ± 0.21 (5)	0.72 ± 0.03	1.45 ± 0.04[a]
	50	1.00 ± 0.10 (8)[a]	0.73 ± 0.04	0.84 ± 0.04[a]
NEA	10	2.50 ± 0.63 (7)[a]	0.80 ± 0.08	1.65 ± 0.17[a]
	50	0.95 ± 0.10 (5)[a]	0.80 ± 0.04	0.83 ± 0.13[a]
NPA	20	1.10 ± 0.14 (12)[a]	0.71 ± 0.04	2.50 ± 0.29
	50	0.60 ± 0.04 (12)[a]	0.70 ± 0.01	0.52 ± 0.04[a]
NBA	20	1.20 ± 0.08 (8)[a]	0.81 ± 0.03	0.95 ± 0.05[a]
	100	0.60 ± 0.07 (8)[a]	0.70 ± 0.06	0.56 ± 0.04[a]
NNDEA	10	0.90 ± 0.05 (7)[a]	0.80 ± 0.06	1.50 ± 0.30[a]
	50	0.55 ± 0.03 (7)[a]	0.70 ± 0.04	0.90 ± 0.13[a]

[a] Differs significantly from control ($p < 0.01$).

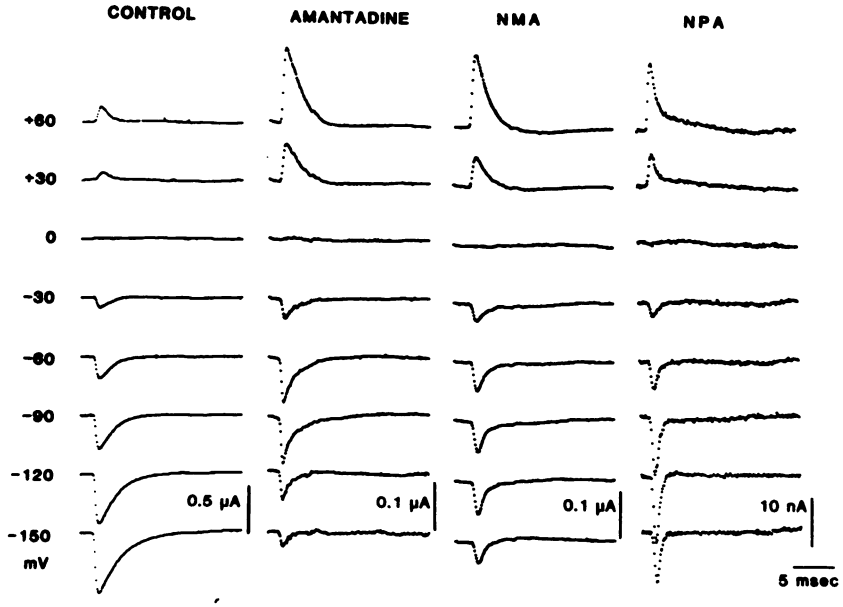


FIG. 5. *Digitized computer output of EPCs at various membrane potentials (from −150 to +60 mV) after exposure to amantadine, NMA, and NPA*

Each series of EPCs was obtained from a single surface fiber of the frog sartorius muscle either before (control) or during exposure for 30 to 60 min to amantadine (100 μM), NMA (20 μM), or NPA (50 μM). Note the different calibrations for control and for drug-treated fibers. Only with NPA is there a doubled-exponential decay in the falling phase of the EPC at positive membrane potentials. The sequence of EPCs began at −50 mV and progressed first toward negative values and then to positive values, returning to −50 mV using 3-sec conditioning steps of 10 mV each.

+0.7 ± 0.04 V^{-1}. Like amantadine, NMA (Fig. 7B) and NEA (Fig. 8B) also reduced the voltage sensitivity of the falling phase of the EPC and changed the slope of the relationship between τ_{EPC} and membrane potential such that 10 μM NMA gave a slope of −0.6 ± 0.03 V^{-1}; 50 μM, +1.4 ± 0.18 V^{-1} (Fig. 7B). Similarly, 10 μM NEA generated a slope of −0.3 ± 0.05 V^{-1}; 50 μM, +1.5 ± 0.09 V^{-1}. NBA and NNDEA reduced the voltage sensitivity of the falling phase of EPC but did not reverse the slope at positive membrane potentials. In the presence of 20 μM and 100 μM NBA, the slopes were −0.6 ± 0.01 V^{-1} and +0.5 ± 0.08 V^{-1}, respectively (Fig. 9B). The same slopes for 10 μM and 50 μM NNDEA were −1.0 ± 0.08 V^{-1} and −0.4 ± 0.01 V^{-1}, respectively. In contrast, in the presence of NPA, the EPC decay became independent of the membrane potential at negative membrane potentials, whereas it remained markedly voltage-sensitive at positive membrane potentials: a double-exponential function appeared consisting of an initial phase of rapid decay, followed by a second phase of sustained decay (Fig. 8B). The normal EPC decayed as a single exponential function of time over its falling phase and is in agreement with previous reports (8, 27, 30). A single exponential decay which was faster than control was observed with all of the concentrations of amantadine and its analogues used (except NPA) and membrane potentials examined. The falling phase of the EPC in the presence of NPA at positive membrane potentials, on the other hand, showed an initial phase which decayed faster, like other analogues, and a terminal phase which decayed much slower than amantadine or any of its analogues (Figs. 5, 8, and 10). The initial phase had a mean τ_{EPC} of 0.59 ± 0.07 msec and contributed about 75% to the total EPC decline. The terminal phase was quite pronounced, and decayed with a τ_{EPC} of 3.88 ± 0.97 msec. It was assumed that the biphasic decay represented two parallel first-order processes occurring at different rates (31). Since the terminal phase was exponential, it appeared likely that this component was present at zero time, although obscured by the faster process. The terminal phase was therefore extrapolated back to zero time and subtracted from the apparent initial phase. This resulted in a straight line with a steeper slope and was used to compute the half-time of the initial phase. Figure 10 shows the resolution of the two components of decay at +60 mV in the presence of 50 μM NPA.

TABLE 4

Comparison of the IC_{50} for blockade of the indirect twitch and end-plate current (EPC) at −90 mV in the frog sartorius muscle and K_i values on the binding of [^{3}H]H_{12}-HTX to channel sites of the ACh receptor in electric organ of Torpedo ocellata obtained for amantadine and analogues with their respective antiviral activity

Drug	Indirect twitch tension IC_{50}	Peak EPC IC_{50}	[^{3}H]H_{12}-HTX binding K_i	Antiviral activity[a] AVI_{50}
	μM	μM	μM	mg/kg
Amantadine	130	64	60	4.6
NMA	15	42	30	3.3
NEA	10	19	15	3.7
NPA	10	<1.0	40	8.0
NBA	40	<2.0	40	16.0
NNDEA	10	<0.5	15	2.9

[a] The antiviral dose$_{50}$ (AVI_{50}) is the amount of compound which causes a 3.2-fold decrease in the infectivity of a standard 20-LD_{50} dose of infecting virus (influenza A S-15) to mice (data from ref. 26). Data for peak EPCs are only approximate.

Effects on ligand binding to Torpedo membranes.

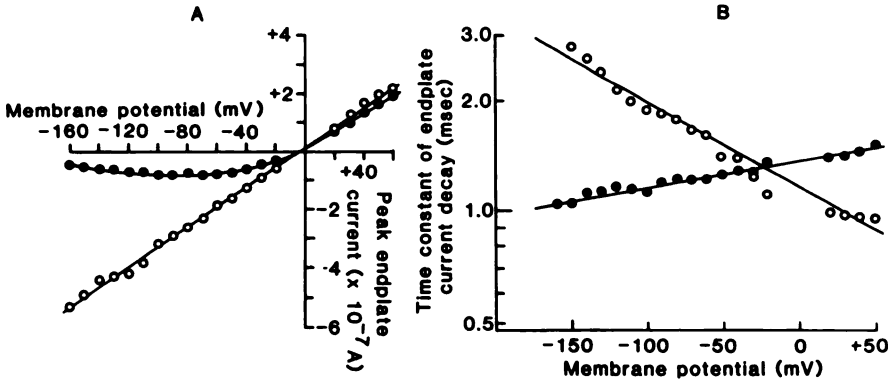


FIG. 6. *Effect of amantadine on the relationship between membrane potential and the peak amplitude of the EPC (A) and the time constant of EPC decay (B)*

Records were made between 30 and 60 min after the addition of amantadine (100 μM) to the bathing solution. Each *symbol* in A and B represents the mean of 10–15 fibers of three muscles. The standard error of the mean was less than 5% of the mean. ○, Control; ●, amantadine.

Neither amantadine nor its *N*-alkyl-substituted analogues inhibited the binding of [^{3}H]ACh (1 μM) to the ACh receptor of *Torpedo* electric organ membranes even at concentrations up to 100 μM. On the other hand, most of them inhibited binding of [^{3}H]H$_{12}$-HTX (2 nM) to the ionic channel sites of the ACh receptor (Fig. 11). Amantadine was the least potent, with a K_i of 60 μM, and NEA and NNDEA were the most potent (Table 4).

DISCUSSION

The present results demonstrate that, like amantadine, its *N*-alkyl-substituted analogues block neuromuscular transmission by interacting with the ionic channel of the ACh receptor. They decrease MEPP amplitude (Table 2) without affecting quantal content, spontaneous MEPP frequency, or resting membrane potential. They depress the peak EPC amplitude in a concentration-dependent manner and cause nonlinearity in the current/voltage relationship (Figs. 6A–9A) and affect the EPC decay phase (Figs. 6B–9B). They also inhibit the binding of [^{3}H]H$_{12}$-HTX to the channel sites of the ACh receptor of *Torpedo* electric organ (Fig. 11) but not that of [^{3}H]ACh to the receptor sites. The apparent decrease in MEPP frequency that has been observed is not a presynaptic effect, but rather a consequence of the block or decrease in MEPP amplitude below the noise level of the recording system (50–100 μV) and due to the postsynaptic effect of these compounds (Table 2), as was previously shown for amantadine (19).

The gross correlation between the potency of the compounds blocking the indirectly elicited muscle twitch and depressing peak EPC amplitude (at −90 mV) and their inhibition of [^{3}H]H$_{12}$-HTX binding to the channel sites is relatively good. An increase in the length of the *N*-alkyl group confers greater potency, in general, for each of these parameters (Table 4). That NBA is less potent than NPA in inhibiting the indirectly elicited muscle twitch, but is as potent in inhibiting [^{3}H]H$_{12}$-HTX binding (Table 2) may be due in part to the different species compared (frog versus *Torpedo*). *N*-Methylating amantadine, for example, increases its potency in blocking the indirect twitch nearly 10-fold, whereas the K_i for the

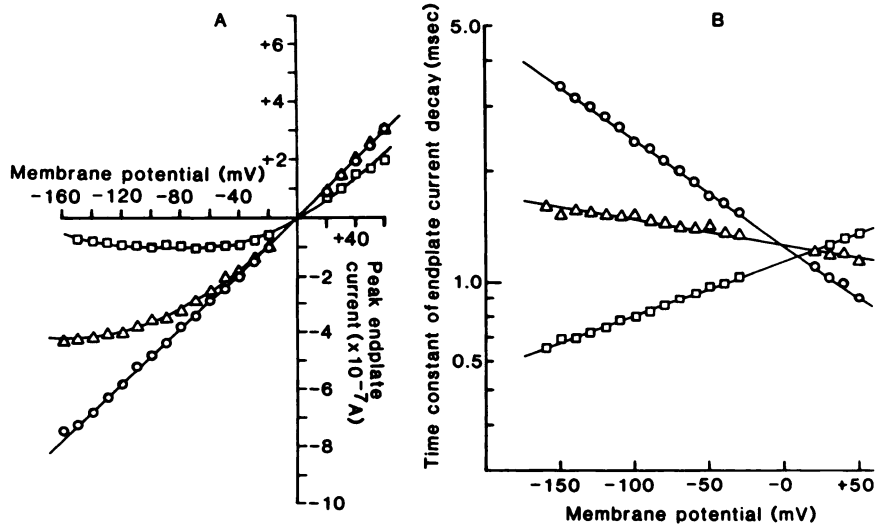


FIG. 7. *Effect of NMA on the relationship between membrane potential and the peak amplitude of the EPC (A) and the time constant of EPC decay (B)*

Records were made between 30 and 60 min after the addition of NMA to the bathing solution. Each *symbol* in A and B represents the mean of 10–15 fibers of three muscles. The standard error of the mean was less than 5% of the mean. ○, Control; △, 20 μM NMA; □, 50 μM NMA.

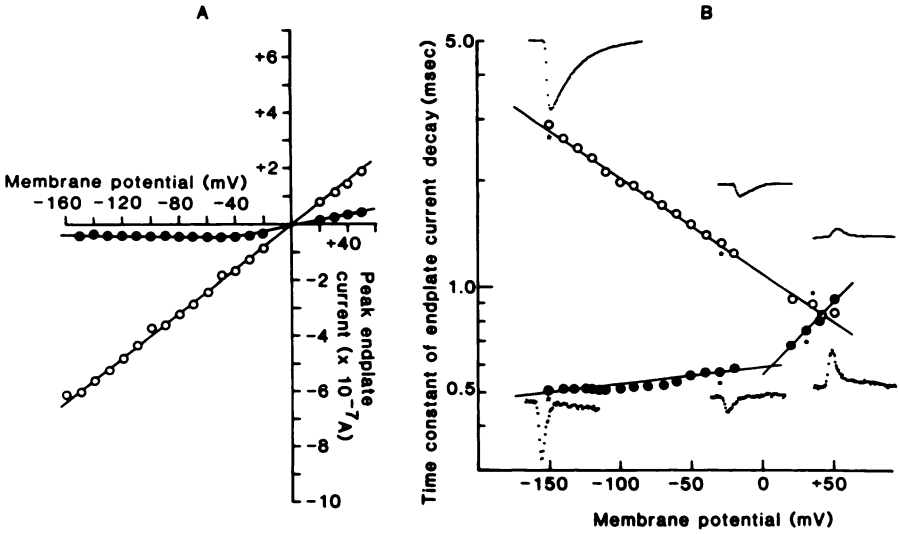


FIG. 8. *Effect of NPA on the relationship between membrane potential and the peak amplitude of the EPC (A) and the time constant of EPC decay (B)*

Records were made between 30 and 60 min after the addition of NPA (50 μM) to the bathing solution. Each *symbol* in A and B represents the mean of 10–15 fibers of three muscles. The standard error of the mean was less than 5% of the mean. ○, Control; ●, NPA.

binding of [^{3}H]H_{12}-HTX is reduced to 50%, and that for 50% inhibition of the peak EPC (at −90 mV) by 30%. Increasing the chain length from methyl (NMA) to ethyl (NEA) has similar effects in lowering the concentration for 50% (IC_{50}) inhibition of muscle twitch, for 50% inhibition of the peak EPC amplitude, and the K_i for inhibition of [^{3}H]H_{12}-HTX binding. Although an increase in chain length to propyl (NPA) does not further affect inhibition of muscle twitch, it reduces its potency in inhibiting [^{3}H]H_{12}-HTX binding (even less than NMA). This is not so for the inhibition of peak EPC amplitude with NPA, NBA, and NNDEA. The increase in chain length from amantidine to NPA did, however, increase the potencies of the compounds (Table 4). Adding another methylene group also has a dramatic effect on the depression of the EPC, since NEA depresses the EPC at negative but not positive potentials, whereas NPA depresses it at both positive and negative membrane potentials.

When these activities are compared with the antiviral effects of the compounds, the most effective *N*-alkyl-substituted compound is NNDEA, but amantadine is more potent than NBA and NPA. However, the insertion of a methylene group between the amine and adamantane nucleus (rimantadine) increases its activity against influenza A/Japan 305 virus (32) and doubles its potency on blockade of the indirect twitch and on the EPC as compared with amantadine.[2] Thus, a strong correlation exists between the structural requirements for the neuromuscular blocking action of these compounds and inhibition of the ionic channel of the nicotinic ACh receptor. Strictly comparable values between inhibition of [^{3}H]H_{12}-HTX binding, the IC_{50} for blockade of the twitch, and the IC_{50} for the EPC were not obtained but may relate to the fact that [^{3}H]H_{12}-HTX binding was not tested in the presence of carbamylcholine (i.e., activated species) or that a simple correlation of the binding data with a single electrophysiological parameter cannot be obtained, considering the complexity of effects on channel properties caused by these compounds and the differences among them. However, it does appear that the structural requirements for antiviral activity, as measured by others, is not strictly identical with those for channel action.

The causal factor in twitch potentiation by amantadine and its analogues is prolongation of the falling phase of the action potential induced either by the appearance of a delayed sodium current or a block of sodium inactivation. Since amantadine and its analogues do not depolarize the muscle membrane, block delayed rectification (a measure of potassium conductance), or alter sodium or potassium conductances (Fig. 4 and text), the possibility of the involvement of voltage-sensitive sodium (i.e., tetrodotoxin-sensitive) or potassium channels in the prolongation of the action potential is virtually ruled out. However, prolongation could still be related to a partial block of potassium conductance even though delayed rectification is unaffected. It seems more likely that prolongation of the action potential and twitch potentiation by amantadine and its analogues (Fig. 4; see also Table 4) are related to a block of sodium inactivation in a way similar to that described by Rojas and Armstrong (33) for the internal action of pronase. Since the prolongation of the action potential produced by most of the analogues is greater than that with amantadine (Table 4), it is likely that the length of the *N*-alkyl chain is directly related to the ability to block sodium inactivation. It should be noted here that several secondary amines (e.g., ketamine; ref. 34) and tertiary alkylammonium compounds (e.g., pancuronium; ref. 35) affect sodium inactivation. Whether a correlation also exists between the prolongation of the action potential and the attenuation of EPCs, caused by *N*-alkyl substitution, must await further experimentation.

The twitch tension experiments show that the analogues are up to 13-fold more potent than amantadine in blocking the indirect twitch (Tables 1 and 4; Fig. 3). The

[2] J. E. Warnick, and E. X. Albuquerque, unpublished observations.

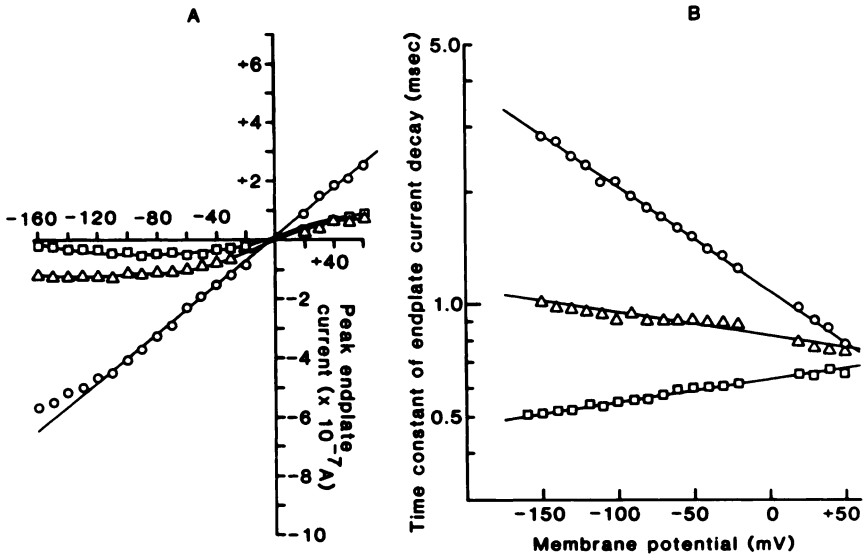


FIG. 9. *Effect of NBA on the relationship between membrane potential and the peak amplitude of the EPC (A) and the time constant of EPC decay (B)*

Records were made between 30 and 60 min after the addition of NBA to the bathing solution. Each *symbol* in A and B represents the mean of 10–15 fibers of three muscles. The standard error of the mean was less than 5% of the mean. ○, Control; △, 20 μM NBA; □, 100 μM NBA.

concentration of amantadine which produces 50% block of the indirect twitch is 130 μM, whereas the 50% inhibitory concentrations for NMA, NEA, NPA, NBA, and NNDEA are 15, 10, 10, 40, and 10 μM, respectively (Table 4; Fig. 3). At concentrations higher than those that inhibit 50% of the indirectly elicited twitch, NPA and NBA potentiate the direct twitch (Table 1).

Amantadine and all of the analogues tested shorten the duration of the EPC in a concentration- as well as a voltage-dependent manner. In the presence of amantadine, NMA, and NEA, the slope of the half-decay time/membrane potential relationship undergoes reversal and the EPC decays faster with hyperpolarization. This effect on τ_{EPC} occurs at both hyperpolarizing and depolarizing membrane potentials, whereas the effect of these drugs on peak EPC amplitude is absent at positive membrane potentials (Figs. 6 and 7). The lengthening of τ_{EPC} induced by amantadine, NMA, and NEA at positive membrane potentials suggests that these drugs remain bound to the ionic channel at positive potentials and generate a large population of conducting channel species, thus explaining the reversal of the slope seen in the presence of the drugs. On the other hand, NBA and NNDEA differ from amantadine, NMA, and NEA in that they do not affect τ_{EPC} at positive potentials (Figs. 5 and 9). Similar shortening of the falling phase without alterations of its exponential

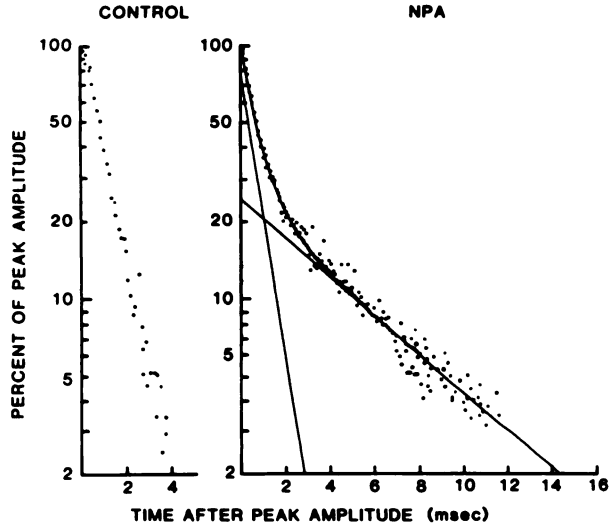


FIG. 10. *Effect of NPA on the decay phase of the EPC recorded at +60 mV*

The data shown are computer records of the falling phase of EPCs from two individual fibers expressed as a percentage of the peak amplitude of the EPC. The lines drawn are computer-generated curves for the first and second phases of decay. The terminal phase was extrapolated back to zero time and subtracted from the apparent initial phase to yield a straight line with a steeper slope than the apparent initial phase. These lines were used to determine the half-decay times of the two components.

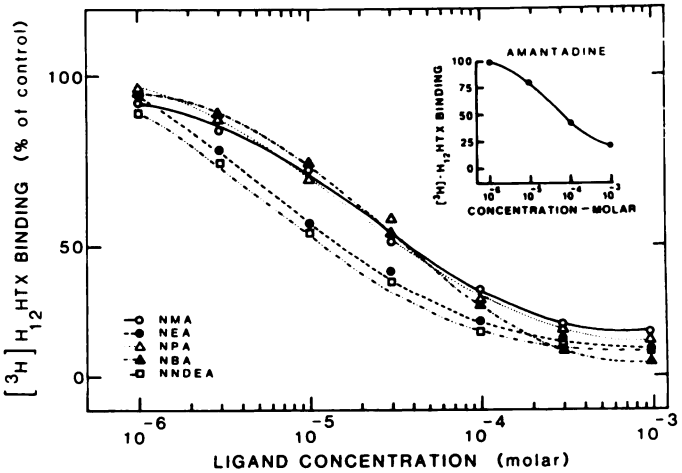


FIG. 11. *Effect of amantadine and analogues on the specific [³H] H_{12}-HTX (2 nM) binding to ionic channel sites of the ACh receptor in Torpedo electric organ membranes plotted as a function of the concentration of amantadine or its N-alkyl analogue*

The curve for amantadine is shown separately as an *inset*. Each experiment was run in triplicate, and standard deviations were less than 10%.

nature was reported for EPCs with histrionicotoxin (9) and miniature EPCs with *n*-octanol (36) and atropine (8).

The analogue NPA differs greatly from amantadine and the other analogues in generating two decay constants at positive potentials (Fig. 8), although the shortening of τ_{EPC} most likely results from an abbreviation of the elementary events (37). The mechanism by which this shortening is achieved remains unclear. NPA depresses peak EPC amplitude, produces a nonlinear current-voltage relationship, and also differs from amantadine and other analogues mainly in the production of a double-exponential falling phase. This is very similar to those produced by scopolamine, but the absence of effect on reversal potential argues against its selective action on one or the other of the ionic species carrying synaptic current.

The biphasic EPC decays produced by NPA suggest that it generates a double-exponential falling phase from two simultaneous first-order processes occurring at markedly different rates. The first phase of rapid decay is nearly independent of membrane potential and thus may be mediated by the same mechanism that produces the voltage-insensitive decay observed with atropine (38). The second phase appears to arise from action on a separate component, which may be similar to the site proposed for short-chain aliphatic alcohols (39). The latter was suggested to act by reducing the dielectric coefficient of the end-plate membrane to cause prolongation in the EPC decline but retention of its normal voltage sensitivity. A similar mechanism has been proposed for the double-exponential decay of scopolamine (38). Thus it is likely that the same mechanism applies to the double-exponential decay of EPC by NPA.

Results of our voltage-clamp experiments and biochemical studies strongly suggest that amantadine and its analogues inhibit neuromuscular transmission through interactions with the ionic channel of ACh receptor. The possible explanation for the different effects of amantadine and its analogues on the EPC amplitude and decay is that the depression of EPC represents blockade of closed end-plate channels, whereas shortening of τ_{EPC} represents blockade of the open channel, both reactions occurring independently and being voltage-sensitive.

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