Proc. Natl. Acad. Sci. USA
Vol. 78, No. 8, pp. 5240–5244, August 1981
Neurobiology

Differentiation of the open and closed states of the ionic channels of nicotinic acetylcholine receptors by tricyclic antidepressants

(neuromuscular synapse/amitriptyline/nortriptyline/endplate currents)

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Contributed by Bernhard Witkop, April 30, 1981

ABSTRACT **The actions of two clinically important dibenzocycloheptane antidepressant drugs, amitriptyline and nortriptyline, were studied on ionic channels of nicotinic acetylcholine (AcCho) receptors at the neuromuscular junction of frog skeletal muscle. Amitriptyline (5–10 μM) and nortriptyline (1–2 μM), like imipramine (5–10 μM), did not react with the nicotinic AcCho receptor but caused a voltage- and time-dependent decrease in the peak amplitude of the endplate current (epc). The time constant of epc decay, however, retained its voltage sensitivity. The voltage- and time-dependent effect of amitriptyline was nonlinear with regard to the current/voltage (*I/V*) relationship. Nortriptyline also had a more pronounced voltage- and time-dependent effect evidenced by a hysteresis loop in the *I/V* relationship of the epc due to the drug's greater potency at more negative potentials. The nonlinearity and hysteresis loop in the *I/V* relationship of the epc was eliminated by the use of 50-msec stepwise changes of the membrane potential. The nonlinearity and hysteresis were due to a time-dependent phenomenon and did not involve previous AcCho receptor activation. The rate constant of the voltage- and time-dependent decrease in epc amplitude was sensitive to the membrane electric field and varied linearly with the membrane potential. Iontophoretically elicited epcs were much more depressed by both drugs than were spontaneous miniature epcs. There was no effect on the time constant of miniature epc decay, single-channel lifetime, or conductance. Thus (as we have pointed out in our histrionicotoxin studies) the primary site of action of these agents presumably is the *activated but nonconducting species of the ionic channel* of the nicotinic AcCho receptor. These agents, particularly nortriptyline, point to several different binding sites of the ionic channel and are suitable tools for the separation of the effects on peak current amplitude from its time constant of decay.**

(Perhydro)histrionicotoxin and phencyclidine produce a blockade of ionic channels associated with nicotinic acetylcholine (AcCho) receptors at concentrations that have no effect on the binding of either AcCho or α-bungarotoxin (1–3). Despite the specificity of these agents for the ionic channels, electrophysiological experiments have revealed multiple binding sites for the agents (4, 5). The blockade of ionic channels in their open conformation by these agents can yield a voltage-dependent decrease in the endplate current (epc) time constant of decay (τ_{epc}) (4). Under certain conditions, however, the voltage- and time-dependent decrease of peak amplitude and consequent hysteresis loop in the current/voltage (*I/V*) relationship of the epc produced by these agents involves the ionic channel in its closed conformation (4–8). Recent studies have provided strong evidence that τ_{epc} may be altered by blockade of the closed channel (7, 8). For example, perhydrohistrionicotoxin yields a plot, $1/\tau_{epc}$ vs. drug concentration, that approaches distinct saturation (7, 8). Such a phenomenon could not be accounted for by the blockade of an open but rather by a closed conformational state of the ionic channel.

Recently, a study has revealed that four tricyclic antidepressant drugs—namely, imipramine, desipramine, amitriptyline, and nortriptyline—have the ability to bind to the ionic channel of the nicotinic receptor and thus inhibit the binding of channel probes, such as phencyclidine and perhydrohistrionicotoxin (9). This inhibition of channel probe binding was enhanced when channel activation was induced with AcCho, a process that probably involved only one site (9). Indeed, one of the tricyclic antidepressants, imipramine, decreased peak epc amplitude of frog sartorius muscle in a concentration-dependent manner (10, 11). Furthermore, with imipramine the *I/V* relationship of the epc became nonlinear, an indication of ionic channel blockade (12, 13), with negligible effect on τ_{epc}. This decrease in peak epc amplitude by an ionic channel mechanism without concomitant effects on τ_{epc} is particularly interesting, for it separates the sites in the ionic channel controlling the peak amplitude of the epc and miniature endplate current (mepc) from their time constants of decay. These results are consistent with a blockade of activated but nonconducting species of the ionic channels. The present study was therefore undertaken to investigate the effects of the dibenzocycloheptane antidepressants amitriptyline and nortriptyline on the nicotinic synapses of frog skeletal muscle in comparison with imipramine (10, 11). Although nicotinic synapses may be only secondary targets for these agents, an understanding of their molecular mechanisms would certainly provide important clues as to their role and primary targets in the central nervous system, including clinical side effects.

MATERIALS AND METHODS

The experiments were performed at room temperature on superficial endplates of cutaneous pectoris muscles of the frog *Rana pipiens*, between December and April. This muscle is particularly suitable for studies in which single-channel recordings are made. The endplate regions are easily visualized and dissection of single fibers of denervated muscles is relatively simple and amenable to "patch" clamp studies (14). Because of the suggestion that τ_{mepc} is changed when these muscles are subjected to glycerol shock (15), we investigated mepcs under control conditions in glycerol-treated and untreated muscles. Glycerol-treated muscles (16, 17) were pinned under slight tension to blocks of Sylgard (Dow) in a 25-ml tissue bath. The muscles were continuously perfused with physiological solution of the following composition (mM): 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 introduced to the bath by changing the perfusion fluid. Micro-

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Abbreviations: AcCho, acetylcholine; *I/V*, current/voltage; epc, endplate current; mepc, miniature endplate current.

electrodes of 3–4 MΩ resistance were positioned at the endplate under visual control and considered to be at the endplate region when endplate potential and miniature endplate potential rise times were less than 1.0 msec. Endplates were maintained under voltage-clamp conditions by using a circuitry similar to that described earlier (18, 19).

epcs. Because our initial experiments with the tricyclic antidepressants (10, 11) disclosed a voltage- and time-dependent effect on epc amplitude, we decided to investigate these voltage- and time-dependent effects by using various membrane potential conditioning sequences (6, 20). Voltage sequence A was similar to that used to examine the effect of histrionicotoxin on epcs in frog sartorius muscles (6). The sequence consisted of 10-mV steps from an original holding potential of −50 mV. The conditioning steps were made sequentially in the depolarizing and then hyperpolarizing directions between the extremes of +50 mV and −150 mV. epcs were elicited at the end of each conditioning step. Three-second conditioning steps were used to avoid any frequency-dependent effects of the drugs. Voltage sequence B was used to test the influence of conditioning step duration on the relationship between epcs and membrane potential. The voltage sequence consisted of voltage excursions of 50-msec duration every 3 sec from a holding potential of −50 mV. The conditioning steps were again made first in the depolarizing and then in the hyperpolarizing direction between the voltage extremes of +50 mV and −150 mV. epcs were evoked 20 msec after the initiation of the conditioning step (20). Voltage sequence C was used to study the actual time-dependent changes in epcs during a sustained hyperpolarizing step that lasted for at least 45 sec or until a steady epc amplitude was obtained (20).

mepcs and epc Fluctuation Analysis. The effects of amitriptyline and nortriptyline on mepcs and epc fluctuations were investigated at room temperature by using cutaneous pectoris muscles treated with 0.3 μM tetrodotoxin. mepc and epc fluctuations induced by iontophoretically applied AcCho were recorded on magnetic tape by a Racal Store 4D FM tape recorder for later analysis on a PDP-11/40 digital computer. mepcs were filtered (1–2500 Hz) by using a Krohn–Hite 3700 bandpass filter and "captured" by a digital oscilloscope (Gould OS4000) before being transmitted to the computer for averaging and analysis.

Power density spectra from AcCho-induced epc fluctuations were obtained from high-gain ac signals after filtering with a Krohn–Hite 3700 bandpass filter (1–800 Hz) (21). Segments of 0.256 sec duration (512 points per segment) were analyzed at a sampling rate of 2 kHz. Digitized records were monitored on a digital storage oscilloscope (Tektronix 603), and those free of obvious electrical artifacts and mepcs were processed by a fast Fourier transform program to obtain power spectra. The power spectra of AcCho-induced noise were obtained from the subtraction of the average of the spectra of baseline samples from the average spectrum in the presence of AcCho. The resulting power spectrum was fitted by a single Lorentzian curve, using a nonlinear fitting program (22). The Lorentzian curve was of the form

$$S(f) = \frac{S(0)}{[1 + (2\pi f\tau_i)^2]}, \quad [1]$$

in which S(0) is the zero-frequency asymptote, $S(f)$ is the spectral density at frequency f, and τ_I is the channel lifetime. The single-channel conductance (γ_I) was calculated according to the formula

$$\gamma_I = \frac{S(0)}{[4\mu(V_m - V_{eq})\tau_I]}, \quad [2]$$

in which τ_I is single-channel lifetime determined from the fitted data, μ is the clamp current measured from a low-gain dc polygraph record, V_m is the clamp potential, and V_{eq} is the equilibrium potential (−15 mV). The half-power frequency (fc) of the Lorentzian curve may also be obtained from the fitted data: $fc = 1/(2\pi\tau_I)$. Cells in which the current did not return to the baseline after AcCho application were discarded. To minimize contamination of the data due to desensitization, noise collection intervals were separated from each other by several minutes.

RESULTS

Time Course and Amplitude of epcs and mepcs. The epc recorded in glycerol-shocked muscles under voltage-clamp conditions at room temperature (22°C) and a holding potential of −90 mV had a rise time of 0.89 msec, a peak amplitude of 0.4 μA, and τ_{epc} of 2.05 msec. Those values are in agreement with epcs recorded in sartorius muscles of *R. pipiens* (23, 24). The peak amplitude under control conditions was voltage sensitive such that the amplitude was linearly related to membrane potential irrespective of the voltage sequence used. Under all conditions the epcs decayed as a single exponential function as described previously (25), the decay of the epc being described by

$$I_{(t)} = I(0)e^{(-t/\tau)}, \quad [3]$$

in which $I_{(t)}$ is the current t msec after the peak, $I(0)$ is the peak amplitude, t is the time in msec, and τ is the time constant of epc decay. τ varied with membrane potential such that the log function of τ_{epc} increased linearly with hyperpolarization, obeying the relationship

$$\tau_{(V)} = Be^{(-AV)}, \quad [4]$$

in which B and A are constants (1.03 msec and 0.00869 mV^{-1}, respectively) and V is the membrane potential. The constants

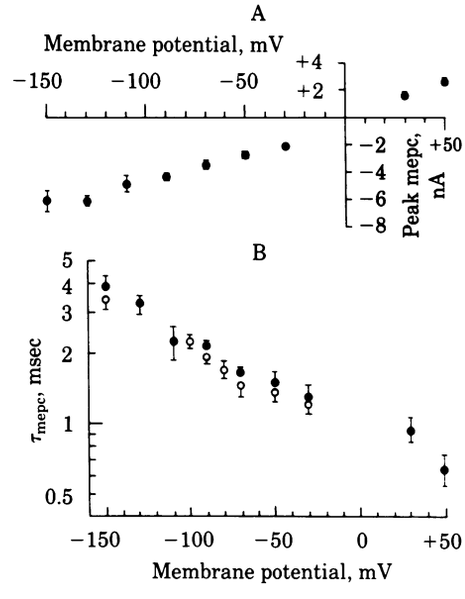

FIG. 1. Effect of membrane potential on mepcs. (*A*) Effect of membrane potential on peak mepc amplitude recorded from glycerol-treated fibers of frog cutaneous pectoris muscle under control conditions (●). (*B*) Effect of membrane potential on τ_{mepc} recorded from glycerol-treated (●) and untreated (○) fibers of cutaneous pectoris muscles. The closed circles represent the mean ± SEM of 20–30 mepcs per fiber in two fibers. The open circles represent the mean ± SEM of 10 mepcs per fiber in six fibers. Note the identical τ_{mepc} recorded in glycerol-treated and untreated fibers.

A and *B* are in close agreement with earlier studies in the sartorius muscle (25). τ_{mepc} recorded from endplate regions of glycerol-shocked muscle fibers and untreated preparations were not significantly different. For example, as seen in Fig. 1, τ_{mepc} had a similar voltage dependence when recorded from glycerol-treated or untreated fibers. The voltage dependence and τ_{mepc} at 0 mV recorded in glycerol-treated cutaneous pectoris muscles were in close agreement with the observations of Gage *et al.* (26). Because the constants *B* and *A* were 1.02 msec and 0.008 mV^{-1}, we cannot support the suggestion that τ_{mepc} recorded from glycerol-treated cutaneous pectoris muscles are prolonged (15). Thus, we decided to use the cutaneous pectoris muscle to investigate the effects of tricyclic antidepressants on ionic channels of the nicotinic receptor.

Effect of Amitriptyline and Nortriptyline on epcs. Exposure of the endplate to amitriptyline for 30–60 min produced a concentration-dependent decrease in peak epc amplitude with a small effect on τ_{epc} (Fig. 2). At −90 mV amitriptyline (5 and 10 μM) decreased the peak epc amplitude to 73% and 21% of the control amplitude, respectively. These concentrations reduced τ_{epc} to 87% and 66% of control, a depression insufficient to explain the decrease in peak epc amplitude by the blockade of open ionic channels. In addition to the decrease in peak amplitude, amitriptyline (10 μM) induced a marked nonlinearity in the epc *I/V* relationship. This nonlinearity proved to be both voltage and time dependent, because it was observed only at potentials more hyperpolarized than −50 mV and was abolished by using 50-msec duration conditioning steps delivered with voltage sequence B. The time-dependent effects of amitriptyline were not fully characterized because they were apparent only when the epc was severely attenuated and, consequently, made nonlinear regression analysis unreliable. These results for amitriptyline were qualitatively similar to those from our earlier studies with imipramine (10, 11).

Nortriptyline also produced a concentration-dependent decrease in peak epc amplitude with less effect on τ_{epc}. During hyperpolarization nortriptyline (1 and 2 μM) at −90 mV decreased the peak epc amplitude to 82% and 51% of the control amplitude, respectively. τ_{epc} was reduced to 87% and 70% of the control value at these concentrations of the agent. Nortriptyline exposure induced a marked voltage- and time-dependent decrease in peak epc amplitude that showed a greater attenuation during depolarization to the holding potential than during hyperpolarization. epc amplitude during the return depolarization to −50 mV was 58% and 29% at −90 mV (Fig. 3). This hysteresis loop in the *I/V* relationship was not apparent in the plot of τ_{epc} vs. membrane potential. The effect of nortriptyline on epc amplitude was qualitatively similar to that of histrionicotoxin and phencyclidine (5, 6). However, unlike the latter two agents, nortriptyline had only a negligible effect on τ_{epc}.

The voltage- and time-dependent decrease in peak epc amplitude induced by nortriptyline could depend on previous AcCho receptor activation or channel openings. It has been shown that channel activation by AcCho increases the affinity of the tricyclic antidepressant binding to the ionic channel (11). The following experiments were performed to test this possibility and to investigate the possible site of action of nortriptyline.

With voltage sequence B and epcs elicited every 3 sec, 20 msec after the onset of 50-msec duration conditioning steps, the *I/V* relationship of the epc was linear over the range −150 mV to +50 mV. The same cell recorded with voltage sequence A showed a marked hysteresis in the *I/V* relationship (Fig. 4). The elimination of the nonlinearity in the *I/V* relationship of the epc

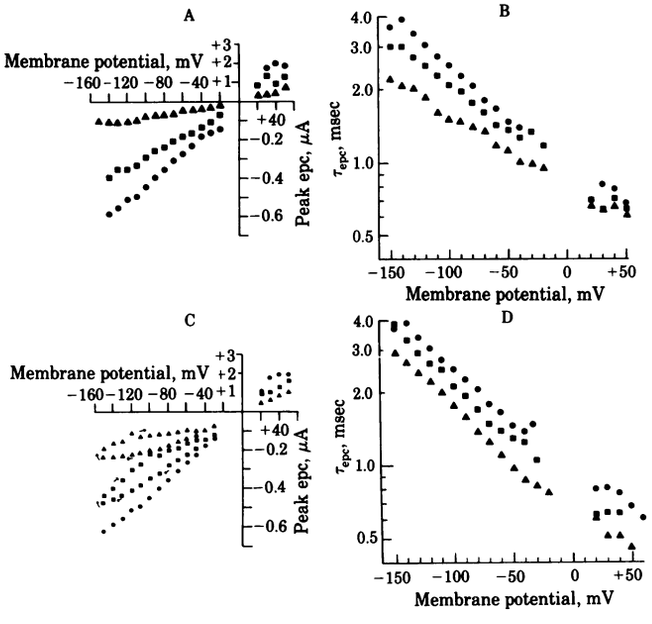

FIG. 2. Blockade of epcs by amitriptyline (*A* and *B*) and nortriptyline (*C* and *D*). (*A* and *C*) The concentration-dependent decrease in epc amplitude. A pronounced hysteresis shown in *B* was observed after treatment with nortriptyline (1 and 2 μM) when the membrane potential was changed from the holding potential at −50 mV to the extremes of +50 mV and −150 mV, using 3-sec conditioning steps, first in the depolarizing and then in the hyperpolarizing direction. This behavior represents a voltage- and time-dependent effect of the agent. Amitriptyline also had voltage- and time-dependent effects that were manifest as a nonlinearity in the *I/V* relationship of the epc (*A*). Both agents produced a small decrease in τ_{epc} shown in *B* and *D* that is insufficient to explain the decrease in peak epc amplitude by an open channel blockade. Note that τ_{epc} retained its voltage dependence after treatment with either agent. The concentrations used in *A* and *B* were: ●, control; ■, 5 μM; ▲, 10 μM; in *C* and *D*: ●, control; ■, 1 μM; ▲, 2 μM. Each point is the mean of at least six fibers; the SEMs were less than 10% of their means.

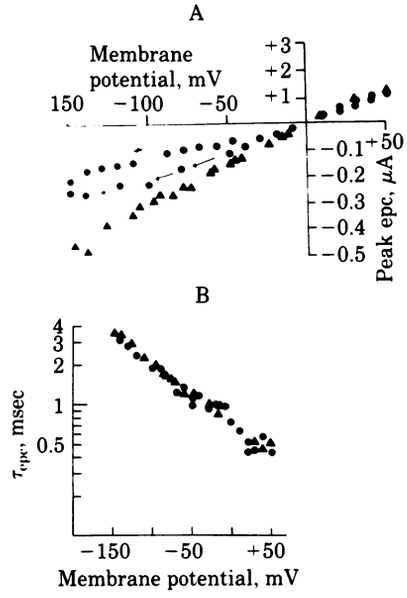

FIG. 3. Time-dependent blockade of epcs by nortriptyline. Membrane potential conditioning steps of 50-msec duration initiated from a holding potential of −50 mV linearized the *I/V* relationship (*A*) of a cell that showed a distinct hysteresis when recorded using membrane potential conditioning steps of 3-sec duration after treatment with nortriptyline (2 μM). τ_{epc} shown in *B* was identical when recorded using 3-sec (●) or 50-msec (▲) duration conditioning pulses.

by using voltage sequence B suggests that the nonlinearity observed when sequence A is used is independent of AcCho receptor activation. In this respect, the results produced by these agents are qualitatively similar to the effects of histrionicotoxin and phencyclidine on epc amplitude (5, 6). τ_{epc} remained unaltered whether voltage sequence A, B, or C was used. It should be noted that neither amitriptyline nor nortriptyline affected the reversal potential of the epc.

Voltage- and Time-Dependent Effect of Nortriptyline. Voltage sequence C was used to investigate the voltage and time dependence of epc amplitude in the presence of nortriptyline. epcs were evoked every 3 sec during a sustained (60-sec) hyperpolarizing conditioning step. To avoid any interference between conditioning steps, only one potential step per cell was made. During these hyperpolarizing potential steps from −50 mV to −100 mV or −150 mV, peak epc amplitude declined over several seconds to a steady level dependent upon membrane potential. The time course of blockade could be fitted by a single exponential function with a rate constant Δ as follows:

$$I_{(td)} = I(0)e^{(-\Delta t)} + K, \qquad [5]$$

in which $I_{(td)}$ is the current at t sec, $I(0)$ is the current at time zero, and K is the steady-state current at the new equilibrium. From nonlinear regression analysis the rate constant Δ at −100 mV and −150 mV in the presence of 2 μM nortriptyline was 0.0335 and 0.068 sec^{-1}, respectively (Fig. 4). The steady-state amplitude K was established approximately 40 sec after the onset of the conditioning step and was 61% and 38% of the zero-time amplitude $I(0)$ (Fig. 4) at −100 mV and −150 mV, respectively. τ_{epc} showed no change during voltage sequence C. After the membrane potential was returned to −50 mV the epc amplitude increased with an exponential time course.

The results thus far suggest that the nonlinearity in the I/V relationship of the epc was not dependent on repeated receptor activation. Further evidence was obtained by stepping the membrane potential from −50 mV to −150 mV for 30 sec in the absence of nerve stimulation. epcs were compared immediately before and after the conditioning step. The results revealed that the epc amplitude was consistently depressed upon return to the prestep potential. Thus the site of action of nortriptyline appeared to be available in the absence of receptor activation by AcCho, providing membrane hyperpolarization was present. Therefore, open channel states appear to be unlikely targets for the action of this agent.

mepc and epc Fluctuation Analysis. The single channel conductance, γ_I, and lifetime, τ_I, under control conditions in cutaneous pectoris muscles at −90 mV were 25 pS and 1.3 msec, respectively, values that are in agreement with those recorded in sartorius muscle (21, 24). Amitriptyline produced a blockade of the AcCho-induced epc at concentrations that had little effect on spontaneous mepc amplitude or τ_{mepc}. For example, amitriptyline (100 nM) produced a large blockade of the AcCho-induced epc with no significant effect on the amplitude or time constant of decay (Fig. 5). This phenomenon, common to both agents, made noise analysis with large concentrations impossible; however, two cells were followed from control through increasing concentrations of the agents to 100 nM nortriptyline and to 200 nM amitriptyline without changes in τ_I or γ_I being observed.

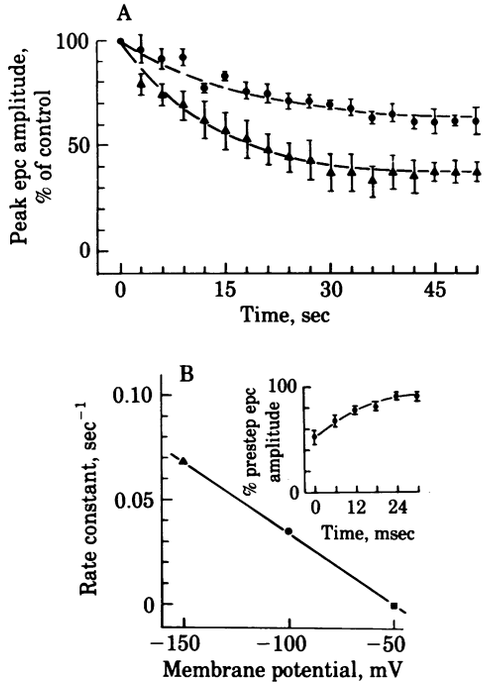

FIG. 4. Time course of the voltage-dependent effects of nortriptyline. (*A*) Time course of the voltage-dependent decrease in peak epc amplitude, expressed as % of control during a sustained hyperpolarizing membrane potential conditioning step to −100 mV (●) or −150 mV (▲). (*B*) Effect of membrane potential on the rate constant of the voltage-dependent blockade of epc amplitude. The recovery of peak epc amplitude upon return to −50 mV is shown in the *Inset*. The concentration of nortriptyline was 2 μM. It should be noted that τ_{epc} remained unchanged during the course of the sustained hyperpolarization. The rate constant of decay and the steady-state current at equilibrium were fitted to Eq. 5 by using nonlinear regression. A minimum of two muscles was used for each curve and at least six endplates were sampled. The vertical bars are SEM.

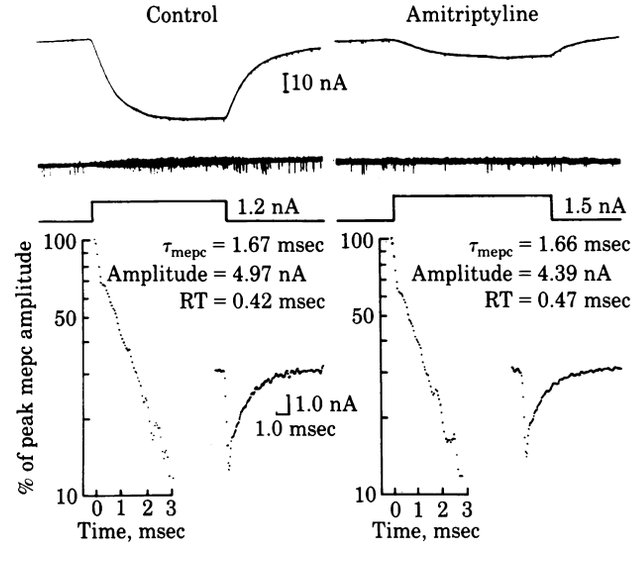

FIG. 5. Differential effect of amitriptyline on spontaneous mepcs and on epcs evoked by iontophoretic application of AcCho. Iontophoretically induced epcs, epc fluctuations, and mepcs were obtained in the absence and presence of amitriptyline at a holding potential of −90 mV and at room temperature. The control traces show the epc induced by a 1.2 nA sec^{-1} iontophoretic current of AcCho and the accompanying epc fluctuations and mepcs. A computer-averaged mepc is shown with its decay phase plotted as a percent of control. Amitriptyline (100 nM) blocked the AcCho-induced epc, whereas spontaneous mepcs were unaffected by this concentration of the agent. A similar differential effect has been obtained with imipramine and nortriptyline, making noise analysis more difficult. However, the results obtained thus far indicate no effect on either channel conductance or lifetime. RT, rise time.

DISCUSSION

The present results show a voltage- and time-dependent blockade of peak epc amplitude after exposure to either amitriptyline or nortriptyline. The nonlinearity in the I/V relationship of the epc appears to represent an interaction of the agents, especially nortriptyline, with a site sensitive to the electric field of the membrane. Evidence for this is provided by the linear dependence upon membrane potential of the rate constants for voltage-dependent epc blockade. The small effects of the agents on τ_I as measured by epc and mepc decay and noise analysis precludes the open channel as the primary site of action of the drugs. A blockade of ionic channels in the closed conformation is the likely locus for epc blockade by these agents. This is in contrast to phencyclidine and histrionicotoxin (1, 4, 27), both of which are thought to interact with both the closed and open conformations of the ionic channel. The open-channel effects of phencyclidine and histrionicotoxin are manifest as a voltage-dependent decrease in τ_{epc}, whereas the closed-channel effects appear as a voltage- and time-dependent decrease in epc amplitude and consequent hysteresis in the I/V relationship to the epc.

The time-dependent decrease in epc amplitude produced by tricyclic antidepressants presumably involves either a decrease in the conductance of the ionic channel after voltage-sensitive binding of the agents or a voltage-sensitive conformational change preventing channel opening. The second possibility best fits the experimental data, because noise analysis revealed no effect on γ_I or τ_I.

Channel activation by AcCho increases the binding affinity of the tricyclic antidepressants to the ionic channel and decreases the K_i for the inhibition of perhydrohistrionicotoxin and phencyclidine binding. The secondary amines were more effective in this respect than the tertiary amines (9). We have been unable to show any depression of the second epc when paired stimuli of various latencies were delivered to the cutaneous pectoris muscle in the presence of the agents. Agonist-activated blockade by tricyclic antidepressants is readily observed with iontophoretic AcCho application to denervated rat skeletal muscle (10, 11).

Receptor blockade is unlikely to account for the effects of the tricyclic antidepressants on epc amplitude, because these agents do not affect the binding of AcCho at the prevailing concentrations (9, 11). Desensitization of the AcCho receptors probably does not account for the actions of the agents, because their voltage- and time-dependent action is evident even in the absence of nerve-evoked AcCho release.

The voltage- and time-dependent blockade of epc amplitude is determined primarily by a set of binding sites that appear to be different from those that control τ_I. In view of the biochemical and electrophysiological evidence, the most likely explanation of the actions of the agents is a *blockade of the activated but nonconducting species of the ionic channel* and thus a voltage-dependent reduction of effective channel number. These antidepressants may prove to be a useful adjunct to the channel probes for the investigation of ionic channel binding, especially to the closed conformation of the nicotinic receptor ionic channels. Although the molecular targets of the tricyclic antidepressant agents on the ionic channel of the nicotinic synapse may be taken as secondary, they nevertheless will provide important clues as to the autonomic, central, and motor disturbances seen in patients treated with tricyclic antidepressants.

The authors are most indebted to Professor Jack Peter Green, Department of Pharmacology, Mount Sinai Medical School, New York, for the initial discussions and ideas regarding the examination of tricyclic antidepressant actions on the nicotinic receptor/ion channel macromolecule. We are grateful to Ms. Mabel A. Zelle for her excellent technical assistance and computer programming and analysis. We also acknowledge Merck Sharp & Dohme Research Laboratories, West Point, PA, for the generous supply of amitriptyline and nortriptyline. This work was supported in part by U.S. Public Health Service Grants NS-12063 and DA-02804 and Army Research Office Grant DAAG-29-78-G-0203.

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