MOLECULAR PHARMACOLOGY, 20:345–355

The Ionic Channel of the Acetylcholine Receptor

Regulation by Sites Outside and Inside the Cell Membrane Which Are Sensitive to Quaternary Ligands

L. G. AGUAYO,[1] B. PAZHENCHEVSKY,[2] J. W. DALY,[3] AND E. X. ALBUQUERQUE[1]

Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201, Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029, and Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

Received September 22, 1980; Accepted April 9, 1981

SUMMARY

AGUAYO, L. G., B. PAZHENCHEVSKY, J. W. DALY, AND E. X. ALBUQUERQUE. The ionic channel of the acetylcholine receptor: regulation by sites outside and inside the cell membrane which are sensitive to quaternary ligands. *Mol. Pharmacol.* **20**:345–355 (1981).

The actions of several quaternary molecules on the end plate region of the frog sartorius muscle were studied using bath application or intracellular injection. Tetraethylammonium (TEA), atropine methyl bromide, phencyclidine methiodide (PCP methiodide), piperocaine methiodide, and *N*-methyl piperidine methiodide were injected into the sarcoplasm just beneath the postjunctional membrane and 250–350 μm away from the end plate region. The ability of these agents to depress potassium conductance and prolong the muscle action potential was used as a measure of the efficacy of intracellular drug administration. External application of TEA (50–1000 μM) decreased the peak amplitude of the end plate current (EPC) and its time constant of decay (τ_{EPC}), but this agent and atropine methyl bromide were ineffective when injected internally. PCP methiodide (3–30 μM) and piperocaine methiodide (10–60 μM) had a potent action on EPCs and spontaneous miniature end plate currents (MEPCs) when applied to either side of the membrane. Both agents caused nonlinearity of the peak amplitude and a shortened channel lifetime in spite of the fact that they sense only 6% of the membrane potential at their rate-limiting energy barrier. Internal application of PCP methiodide caused significant depression of the EPC and MEPC peak amplitude and simultaneous shortening of MEPC decay time constant. The decay time constant of the EPC and MEPC in the presence of PCP methiodide and piperocaine methiodide was shorter at less negative (i.e., −60 mV) than at more negative (i.e., −100 mV) membrane potentials. Similar results were obtained with internal applications of piperocaine methiodide. *N*-methyl piperidine methiodide, a quaternary contaminant of PCP methiodide, did not display any effect when it was applied inside the cell. The results indicate that sites controlling the ionic channel of the acetylcholine receptor from the external surface may be significantly different from sites on the internal surface, even though both sites do interact with certain quaternary amines. These findings further indicate that the ionic channel is asymmetrical with the selectivity gate located most likely at the intracellular region of the channel. Finally, it is suggested that the decay time constant of the ionic currents in the presence of tertiary agents may be an average, voltage-dependent result of the actions of the agent inside and outside the cell.

This study was supported in part by United States Public Health Service Grant NS-12063 (to E. X. A.), by National Institute of Drug Abuse Grant DA-02534 (to B. P.), and by United States Army Research Office Grant DAAG-29-78-G-0203 (to E. X. A.).

[1] Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine.

[2] Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York.

[3] Laboratory of Bioorganic Chemistry, National Institutes of Health.

0026-895X/81/020345-11$2.00/0

INTRODUCTION

The current generated by the interaction of ACh[4] with its active sites located at the junctional membrane of the neuromuscular synapse is sensitive to a number of ligands which noncompetitively or allosterically bind to receptor or channel sites, thus altering the amplitude and time course of the ionic current (1–4). For example, atropine, local anesthetics, amantadine, PCP, and HTX modify the voltage dependence of the τ_{EPC} and produce a marked curvature in the current-voltage relationship of the EPC (5). These agents react with the ion channel of the ACh receptor, also termed ion conductance modulator (6). Since such lipophilic drugs and toxins are able to block the end plate channel when applied in the bath, their sites of action are accessible from outside the cell. However, it is unknown whether the binding sites for such agents are on the external surface, within the membrane channel, or in the internal compartment of the cell. Indeed, the possibility exists that such sites may be present at all three of these loci. Most local anesthetics, because they are very lipid-soluble, are of limited value for this type of investigation since they readily permeate membranes (7). Quaternary amine derivatives, however, are very polar and will penetrate lipid phases and cross membranes only to a limited extent. In addition, quaternary amines have a readily measured intracellular effect that is independent of the ACh receptor-ion channel complex and which can serve as a sign that the drug is present within the cell: they block the electrically excitable K^+ channel, thereby prolonging the muscle action potential. Among the quaternary compounds considered most suitable for investigation of intracellular sites associated with the ACh-receptor ionic channel complex were TEA, PCP methiodide, piperocaine methiodide, and atropine methyl bromide. These agents are advantageous for these studies because their tertiary amine analogues react with the ionic channel of the ACh receptor, have potent effects on the EPC when applied externally, and also block the potassium conductance, as revealed by voltage-clamp studies and by effects on the action potential and delayed rectification (8–10).

The objective of the present study is to elucidate the reaction of quaternary agents with sites located in the ionic channel at its intracellular region. The present investigation shows that intracellularly applied TEA and atropine methyl bromide did not significantly affect the EPC characteristics, but that PCP methiodide and piperocaine methiodide induced significant alterations of the EPCs, thus confirming earlier studies by this laboratory (5, 11) and others (12, 13).

METHODS

Electrophysiological techniques. All experiments were carried out *in vitro* at 21–23° on isolated sciatic nerve-sartorius muscle preparations of the frog *Rana pipiens* (3). To eliminate muscle contraction, some preparations were treated with Ringer's solution containing 600 mM glycerol and then washed with normal Ringer's solution. Muscles were mounted under slight tension on a paraffin block which had a plano-convex lens in the center and were perfused with normal Ringer's solution of the following composition (millimolar): NaCl, 115; KCl, 2.0; $CaCl_2$, 1.8; NaH_2PO_4, 1.3; and Na_2HPO_4, 0.7. All drugs were prepared from refrigerated stock solutions.

For intracellular recording, a conventional voltage-clamp circuit was used (3, 4). In some preparations, the prolongation of the muscle action potential, a well known effect of TEA and PCP, served as a criterion for successful drug access to the internal compartments of the cell. Figure 1 shows the electrical arrangement. The procedure for intracellular drug release was as follows. (a) The recording microelectrode was inserted at the end plate region in fibers with membrane potential varying from −60 to −100 mV. It was important to record from cells within this range of membrane potential to avoid major variations in the rising and falling phases of the indirectly evoked action potential. After the recording microelectrode was inserted, the nerve was stimulated to verify the presence of a muscle action potential. (b) The second microelectrode (Microelectrode 2) was then inserted within 25 μm of the recording microelectrode. To assure that the second microelectrode was inside the same cell, a square pulse of 5-msec duration was applied to this microelectrode and the electrotonic potential was detected by the recording microelectrode. (c) The micropipette containing the quaternary agent (Microelectrode 3) was then inserted into the muscle just beneath the ACh receptor-rich junctional region, i.e., at the end plate region, using a procedure identical with that described for Microelectrode 2. Positioning of the microelectrodes was accomplished in some experiments by direct visualization using a Nomarski interference optics microscope as described earlier (14). In this way, the drug-containing microelectrode was inserted between the two other microelectrodes along the length of the end plate. In other experiments, the drug-containing microelectrode was positioned about 150–300 μm from the end plate region. TEA, atropine methyl bromide, PCP methiodide, *N*-methyl piperidine methiodide, and piperocaine methiodide were injected from microelectrodes filled with 2 M (5–15 MΩ), 2 M (10–15 MΩ), 0.01 M (10–35 MΩ), 0.01 M (10–35 MΩ), and 0.001 M (20–30 MΩ) solutions, respectively. Once all three microelectrodes were inside the cell, the nerve was stimulated and control action potentials were recorded. After the voltage clamp was activated using the recording microelectrode (2–4 MΩ) and Microelectrode 2 (2–4 MΩ), a series of end plate currents was recorded at various membrane potentials. The voltage clamp was then turned off, and a switch connecting the drug-containing micropipette (Microelectrode 3) and the second microelectrode was activated to deliver an outward current in reference to Microelectrode 2. Usually a maximum of 1 μA of current was passed through the microelectrodes for 1–2 min. Action potentials were monitored following drug injection. The switch then returned Microelectrode 2 to voltage-clamp condition, leaving Microelectrode 3 inside the cell but disconnected from the circuit, and a second EPC family was recorded. Control

[4] The abbreviations used are: ACh, acetylcholine; PCP, phencyclidine; HTX, histrionicotoxin; EPC, end plate current; τ_{EPC}, time constant of EPC decay; TEA, tetraethylammonium bromide; MEPC, miniature end plate current; τ, channel lifetime; τ_{MEPC}, time constant of MEPC decay.

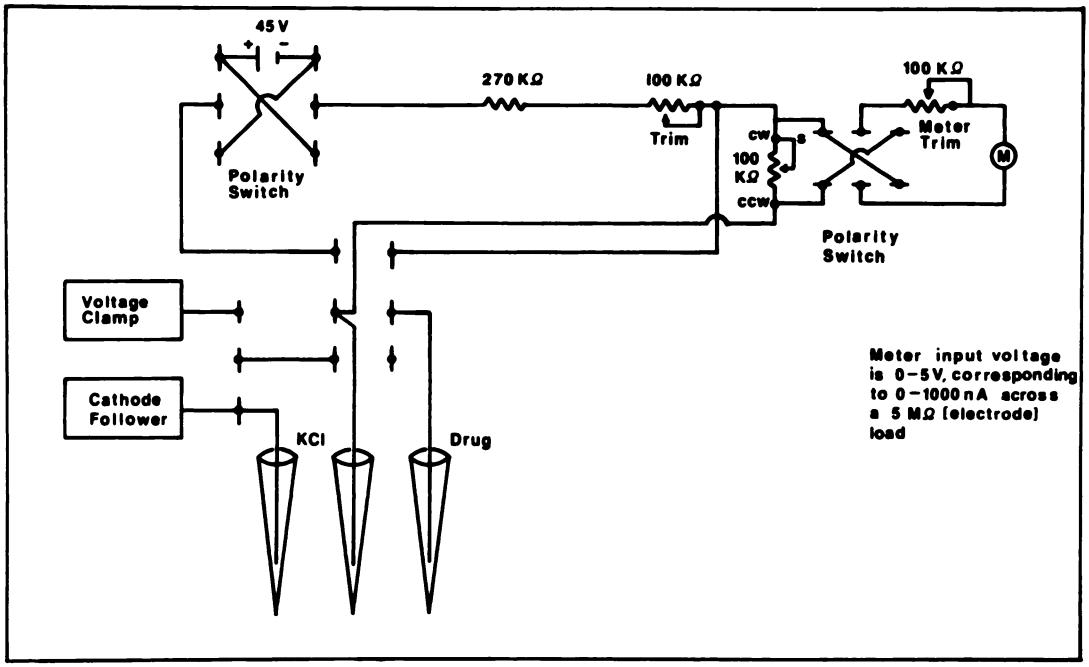

FIG. 1. *Electrical circuit used for injection of drug into the single fibers of Rana pipiens sartorius muscle (see Methods for details)*

conditions for the intracellular applications were achieved by applying a constant 1 μA of current for 2 min to a KCl microelectrode in a location similar to that of the other microelectrode containing the drug. On a few occasions we also used as a control the EPC or MEPC records from an end plate region whose neighboring fiber had the drug-containing microelectrode inserted near the voltage-clamped end plate. These two methods eliminated any biasing and also assured that no leakages of the drugs used were responsible for the intracellular effect observed.

Treatment of EPC and MEPC data. The DC output of a Tektronix oscilloscope, which displayed the nerve-evoked EPC and the membrane potential, was sampled at a rate of 100 μsec per point using an analog to digital converter and analyzed by a laboratory computer (PDP 11/40; Digital Equipment Corporation, Marlboro, Mass.). The decay time constant (τ) of each EPC was determined by linear regression of the logarithms of the digitized decay (20–80%) points against time. Acceptable EPCs had a correlation coefficient of 0.97 or better (3, 15). The MEPCs, generated by spontaneous release of ACh, were recorded with voltage-clamp techniques. MEPCs captured by a digital oscilloscope (Gould Inc., Cleveland, Ohio) were passed through a band pass (1–2500 Hz) filter (Krohn-Hite 3700), before being transmitted to the computer (16). For the MEPC spectra, cells displaying a high MEPC frequency were used. MEPCs were amplified on a high-gain AC channel and recorded on an FM tape recorder (Tandberg 115). The signal was filtered with a band pass filter (1–800 Hz) to exclude high-frequency noise, sampled at 2 kHz, and analyzed in segments of 0.256-sec duration (512 points per segment). The average spectrum of the baseline noise of the cell was subtracted from that obtained from segments containing MEPCs. Spectra of MEPCs were fitted by least squares method to a single Lorentzian for computation of τ according to the formula $\tau = 1/(2\pi fc)$, where fc is the half-power frequency of the Lorentzian (3, 4, 17).

Drugs and toxins. Piperocaine methiodide was synthesized as follows: Piperocaine HCl (Lilly Research Laboratories, Indianapolis, Ind.) was dissolved in water, neutralized with excess ammonia, and extracted into chloroform. After the chloroform layer was dried over sodium sulfate and evaporated to dryness *in vacuo*, the resultant piperocaine (free base) was dissolved in acetonitrile and methylated with a 4-fold excess of methyl iodide at room temperature overnight. The solution was evaporated *in vacuo* to dryness, and the residue was triturated with acetone and then recrystallized twice from methanol-diethyl ether to yield piperocaine methiodide in 80% yield. The product was analytically and chromatographically pure. The limit of chromatographic detecton of piperocaine in the sample was estimated at about 0.05%.

PCP methiodide was synthesized from PCP (free base) and methyl iodide (2-fold excess) in acetone-diethyl ether. The crystalline precipitate, formed after 3 days at room temperature, was examined by high-pressure liquid chromatography (reverse phase; acetonitrile/water, 80:20). A single contaminant was found. Isolation of the by-product and comparison with authentic sample identified it as *N*-methyl piperidine methiodide. Repeated attempts to separate the *N*-methyl piperidine methiodide from PCP methiodide by crystallization were unsuccessful. The relative amounts of the by-product, in recrystallized (methanol-ether) sample, were found to be 10–15% from the integration ratio of the aromatic and the aliphatic region in the proton NMR spectrum.

TEA bromide was purchased from J. T. Baker Chemical Company (Phillipsburg, N. J.) and atropine methyl bromide from Sigma Chemical Company (St. Louis, Mo.). PCP methiodide, piperocaine methiodide, and *N*-methyl piperidine methiodide were synthesized in our laboratories.

RESULTS

External and internal application of PCP methiodide and piperocaine methiodide to the junctional region of neuromuscular synapses. External application of PCP methiodide at concentrations varying from 3 to 30 μM caused significant depression of the peak amplitude of the EPC and induced a marked nonlinearity in the current-voltage relationship (Fig. 2). As shown in Fig. 2A, the nonlinearity of the current-voltage relationship in control and after the addition of PCP methiodide to the bathing solution at a concentration of 3, 10, or 30 μM become more marked as the membrane potential was shifted toward hyperpolarizing levels. Apparently, PCP methiodide was able to depress the peak amplitude of the EPC at concentrations as low as 3 μM. As was found with PCP, the amplitude of the EPC in the presence of PCP methiodide showed a marked upward curvature and a region of negative conductance where the EPC amplitude decreased in spite of increases in driving force. The possibility thus exists that PCP methiodide caused this nonlinearity in the current-voltage relationship via a mechanism similar to that of PCP (4, 5), i.e., PCP methiodide reacts with the channel which will fail to open upon activation by ACh. If the reaction of PCP methiodide resembles that of its parent compound, PCP, the channel would react with the former slowly relative to the time of shifting the membrane potential. Reduction of the conditioning step from 3 sec to 10 msec would then make the current voltage relationship linear. Using this technique, PCP methiodide induced a time-dependent effect (for details of this technique see refs. 4 and 5), i.e., when the short conditioning pulse of 10 msec was used, the nonlinearity of the peak amplitude of the EPC induced by the agent during the long conditioning pulse of 3 sec was abolished. However, in contrast to PCP, the quaternary agent PCP methiodide did not cause a hysteresis loop in the current-voltage relationship, a phenomenon which is most likely due to a slower equilibration to potential changes (4, 5). In contrast to its tertiary analogue, PCP, and HTX, which may have several reactive sites along the channel (4, 5), PCP methiodide may not penetrate to deeper portions of the ionic channel (see below). Concomitantly with a marked depression of the peak amplitude, PCP methiodide caused acceleration of τ_{EPC} (Fig. 2B). The rise time of EPC and MEPC was not significantly affected by PCP methiodide (30 μM). PCP methiodide produced significant acceleration of the τ_{EPC} and τ_{MEPC} and, similarly to PCP, significantly reduced the dependence of τ on membrane potential (Fig. 2B, 3A). At −90 mV, τ_{EPC} (Fig. 2B) and τ_{MEPC} (Fig. 3A) underwent a significant shortening. The action of PCP methiodide on τ was concentration-dependent, an effect similar to that of PCP (4). At a concentration of 30 μM of PCP methiodide applied externally, τ_{EPC} was reduced to about 0.85 msec at −150 mV, an effect similar to that seen with PCP. Throughout these experiments, the EPC decay remained a single exponential function of time. The effect of PCP methiodide on the peak amplitude of the EPC and τ_{EPC} produced a slight discrepancy between acceleration of the decay time constant versus the effect on peak amplitude. In fact, at 10 μM, the plot of per cent

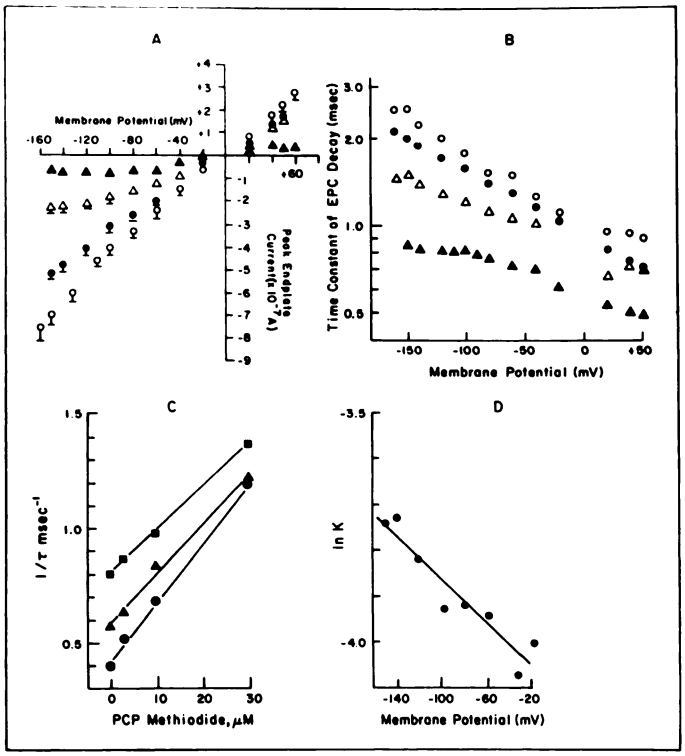

FIG. 2. *Current-voltage relationships of the EPC peak amplitude (A), and semilogarithmic plot showing the time constant of decay of the EPC versus membrane potential (B) in control (○) and in the presence of various concentrations of PCP methiodide*

The drug was added to the physiological solution (external application) at the following final concentrations: 3 (●), 10 (△), and 30 μM (▲). Each *symbol* represents the mean ± standard error of the mean of 9–14 fibers from six or more muscles. In *C* is shown a plot of τ^{-1}, obtained from EPC decays (B), versus PCP methiodide concentrations at −150 (●), −100 (▲), and −40 mV (■). The slopes of these lines, in units of $msec^{-1}$ and μM^{-1}, are plotted on a semilogarithmic scale against membrane potential in *D*. The *straight line* in *D*, obtained by linear regression analysis from at least eight different measurements, was significantly different from zero ($p < 0.005$).

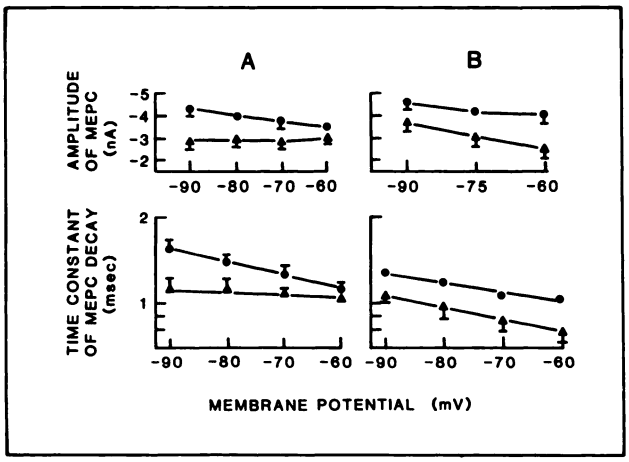

FIG. 3. *Effect of external (A) and internal (B) applications of PCP methiodide on spontaneous MEPC*

Both *A* and *B* show the peak amplitude and time constant of decay (τ) of the MEPC under control condition without any drug (●), in the presence of bath-applied 10 μM PCP methiodide (▲) (*A*), and after internal injection (1 μA, 1.30 min) (*B*).

peak amplitude of the EPC at most of the membrane potentials investigated showed a significant depression of the peak amplitude in contrast to τ_{EPC}.

It is of importance to analyze the data shown in Fig. 2B to verify the location of the PCP methiodide energy barrier within the membrane electrical field (18). Indeed, a number of large organic cations can effectively block the end plate channel when applied externally, thus suggesting an available outer surface binding site on the channel for the drugs. The activated conducting species of the channel, AR^*, is assumed to react by two routes to yield nonconducting species, in a similar fashion to that described by the sequential model (19):

$$AR \rightleftharpoons AR^* \overset{D_{k_3}}{\rightleftharpoons} AR^*D$$

The back reaction, $AR^*D \rightarrow AR^* + D$, is neglected because only single exponential decays were seen. An appreciable back reaction would produce double exponential decays (5). Under this condition, the time constant for the EPC decay is described by the expression $\tau = (\alpha + [D]k_3)^{-1}$, where k_3, the second-order rate constant for drug binding, is the slope of the plot $1/\tau$ versus $[D]$ (Fig. 2C) (5). The fraction of membrane potential sensed by externally applied PCP methiodide can affect the rate constant for the drug binding to its active site on the ionic channel as follows:

$$k_3 = K_0 \exp\left(\frac{-neV\delta}{kT}\right)$$

where K_0 is the rate constant at 0 mV, ne is the charge of the agent, V is membrane potential, k is Boltzman constant, and T is absolute temperature. The value of δ the fraction of the membrane potential sensed by the drug cation can be calculated from the slope of a plot of ln K versus V, as shown in Fig. 2D. Although susceptible to uncertainties, this analysis suggests that PCP methiodide senses only 6% of the membrane potential (5), an observation which differs markedly from procaine (1) and QX-222 (20), which sense the membrane potential midway through the membrane electric field.

Another quaternary agent, piperocaine methiodide, applied externally caused nonlinearity of the peak amplitude of the current-voltage relationship (Fig. 4A) of the EPC and shortened τ_{EPC} (Fig. 4B) in a concentration-dependent manner (Fig. 4A and B). Similarly to PCP methiodide, the effect of piperocaine methiodide was also initially analyzed for its reaction with external sites on the ionic channel. The results shown in Fig. 4 are qualitatively similar to those produced by PCP methiodide. Similar to PCP methiodide, piperocaine methiodide also appears to sense the membrane potential by 6% (Fig. 4C and D) at its binding sites, thus suggesting that the agent reacts at the same site as does PCP methiodide.

Since it has been demonstrated that the ACh receptor ionic channel macromolecule is a structural protein imbedded into the lipid membrane and extending above the extracellular surface by about 55 Å (21, 22), it is possible that the binding sites for both PCP methiodide and piperocaine methiodide are located above the transmembrane electric field.

When PCP methiodide (0.01 M) was released from a micropipette inserted inside the cell at the junctional region, a significant depression of the EPC peak amplitude and acceleration of the decay time constant was seen to occur at negative potentials predominantly at the region of −90 to −30 mV. Such an effect of internal application of PCP methiodide is shown in Fig. 5. Figure 5 shows an indirectly elicited muscle action potential and a family of EPCs prior to release of PCP methiodide into the cell. Injection of PCP methiodide for 2 min using a 900-nA pulse from the micropipette filled with the agent caused a significant prolongation of the half-decay time of the action potential (Fig. 5), an effect which was slowly reversible after about 45 min. This prolongation of the action potential appears to reflect a blockade of potassium conductance, thus serving as a monitor of the presence of the agent, which is able to affect the spike activity, the EPCs, and the MEPCs at similar concentrations. When applied internally, the effect of PCP methiodide on the EPC peak amplitude (Fig. 5) at −120 and −20 mV resembled that of a 10 μM concentration of the

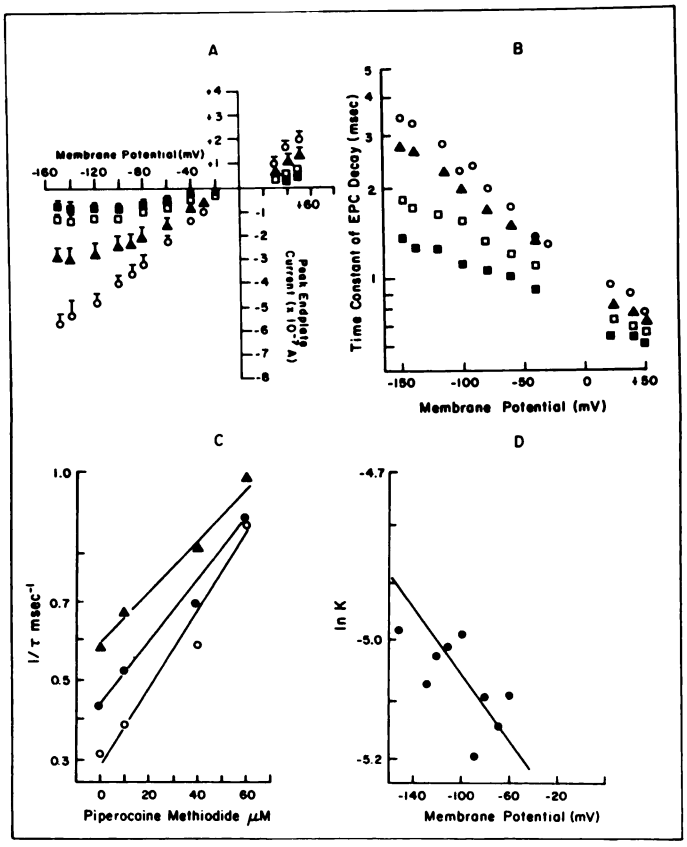

FIG. 4. *Current-voltage relationships of the EPC peak amplitude (A) and semilogarithmic plots illustrating the time constant of decay of the EPC versus membrane potential (B) recorded under control condition* (○) *and in the presence of piperocaine methiodide (external application): 10* (▲), *40* (□), *and 60 μM* (■)

Each *symbol* represents the mean ± standard error of the mean of 7–12 fibers from five or more muscles. In *C*, the inverse of time constant of EPC decay versus concentration of piperocaine methiodide is shown at membrane potentials of −140 (○), −100 (●), and −60 mV (▲). The slopes of these lines, in units of $msec^{-1}$ and μM^{-1}, are plotted on a semilogarithmic scale versus membrane potential in *D*. The straight line in *D*, obtained by linear regression analysis from at least 10 different measurements, was significantly different from zero ($p < 0.05$).

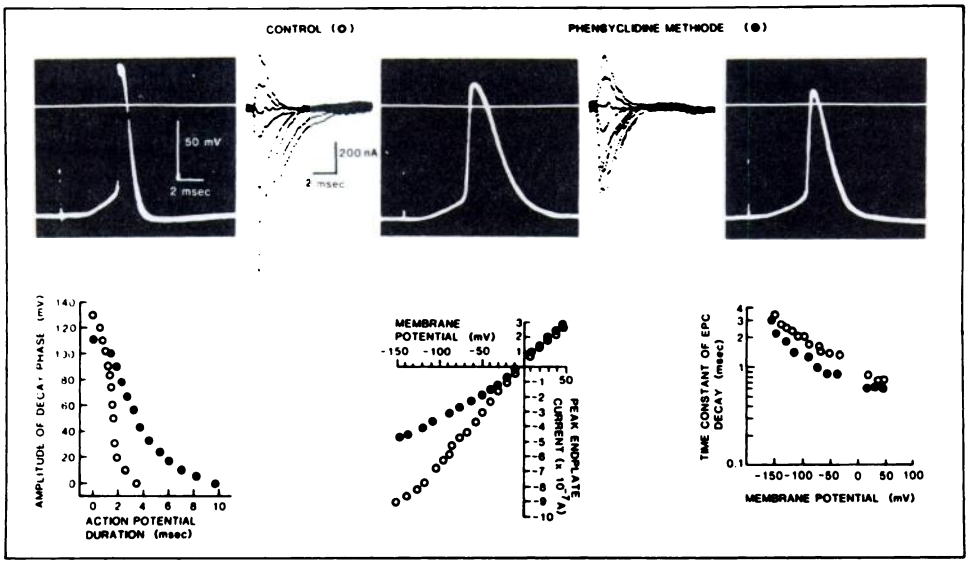

FIG. 5. *Effects of intracellular injection of PCP methiodide on the electrical excitability and ionic channel of the ACh-receptor of the frog sartorius junctional membrane*

An indirect action potential (*upper left panel*) and a family of EPCs (*upper left family*) were generated under control condition. The time course of the action potential is plotted as amplitude versus time after the peak in the *lower left graph* (○). Thereafter, a current of 900 nA was passed for 2 min via a micropipette containing 0.01 M PCP methiodide. Significant prolongation of the action potential [*upper center panel* and *lower left graph* (●)] was a sign of successful internal injection. In contrast to control (○) condition, when the second family was elicited (the *upper right family* and the ● in the *plots*), marked alterations in the current-voltage were observed (*lower center panel*). The *upper right panel* shows that the decay phase of the indirect elicited action potential was still prolonged at 30 min after the internal injection of PCP methiodide. The relationship between time constant of the EPC decay and membrane potential was also altered. Notice that the acceleration of τ was larger at less negative membrane potential.

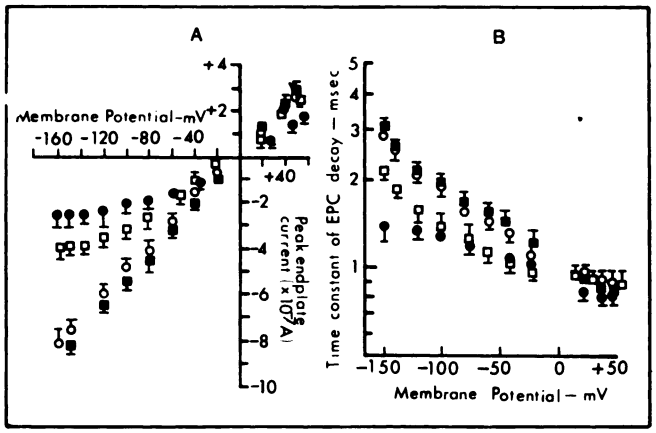

FIG. 6. *Comparison between the external and internal cellular application of PCP methiodide*

Peak EPC amplitude (*A*) and time constant of decay of the EPC (*B*) are plotted against membrane potential. 10 μM PCP methiodide applied externally (●); and control (○); (□), PCP methiodide applied internally and its control where no drug was released inside (■). The *symbols* represent mean values and standard error of the mean from four to eight end plates.

agent applied extracellularly (Fig. 6A). Internally applied PCP methiodide has, at very negative potentials, i.e. −150 mV, a smaller effect on the τ_{EPC}. This reflects an apparently lower reaction with the active sites regulating the lifetime of the ionic channel (Fig. 6B). When PCP methiodide was applied extracellularly and the membrane potential was varied from −60 to −150 mV, the shortening of τ_{EPC} was more evident (compare Fig. 2B with 6B and 3). A further observation of Figs. 5 and 6 shows that when the family of EPCs was generated within 10 sec after termination of drug injection, the current-voltage relationship exhibited a reduced slope conductance and nonlinearity in the hyperpolarized region. The alterations were also seen to occur in the MEPCs as shown in Fig. 3B, where internal application of PCP methiodide disclosed an effect on MEPC peak amplitude, and τ similar to that seen on the EPC. Indeed, τ_{MEPC} (Fig. 3B) was shortened more at −60 than at −90 mV. The MEPC spectra obtained at −60 and −90 mV are shown in Fig. 7. At −60 mV, the control MEPC spectra had a τ of 1.8 msec, and after internal application of PCP methiodide τ became significantly shorter, with a value of 1.35 msec. At −90 mV, however, the control value was τ = 2.0 and 2.2 msec in the presence of PCP methiodide, thus suggesting that the agent becomes partially dissociated from its active sites as the membrane potential is driven to more negative values.

An important observation which leads to the conclusion that the quaternary form of the local anesthetic piperocaine methiodide, and of PCP methiodide, reacts with the ionic channel of the ACh receptor, concerns the absence of effects on EPC and MEPC when the drug-containing pipette was located a distance greater than 250 μm (Fig. 8B) from the end plate region (contrast with the effects illustrated in Fig. 8A). In addition, the externally applied quaternary amines have their maximal effect at the most negative potentials, whereas in contrast the internally applied agents have their greatest effect at less negative potentials (see Figs. 6 and 8). Indeed, when these agents were applied inside the junctional region, piperocaine methiodide produced significant depression of the peak amplitude of the EPC, culminating with

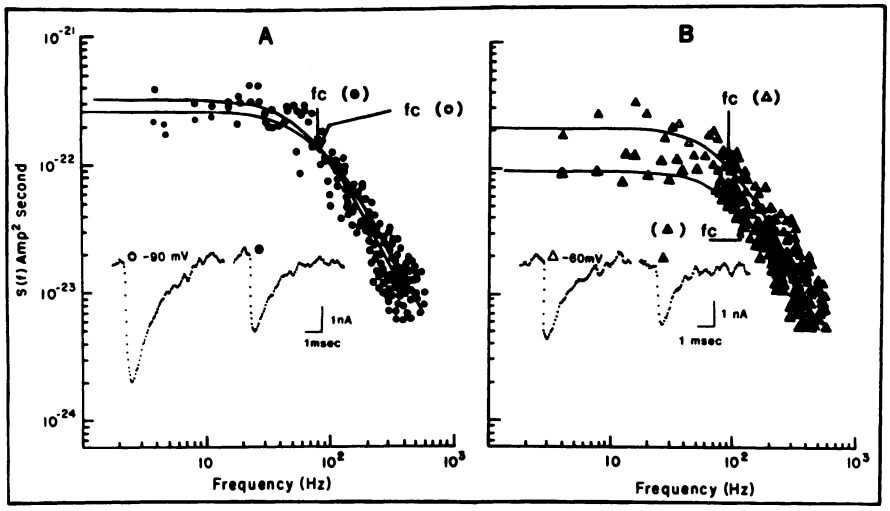

FIG. 7. *Effect of intracellular application of PCP methiodide on MEPCs and their spectra*

A was obtained at −90 mV (A) in control (○) and after intracellular injection of PCP methiodide (1000 nA, 1.30 min) (●). B shows control (△) and after injection (▲) obtained at −60 mV. Spectra are averages obtained for two to seven MEPCs. The time constant of decay ($\tau = 1/(2\pi fc)$) at −60 mV was 1.8 msec and after PCP methiodide internal injection was 1.35 msec. At −90 mV, however, a shortening of τ upon drug injection was not observed, i.e., $\tau = 2$ msec for control condition, and 2.2 msec after internal injection of PCP methiodide. Typical spontaneous MEPCs under these circumstances are shown as *insets*.

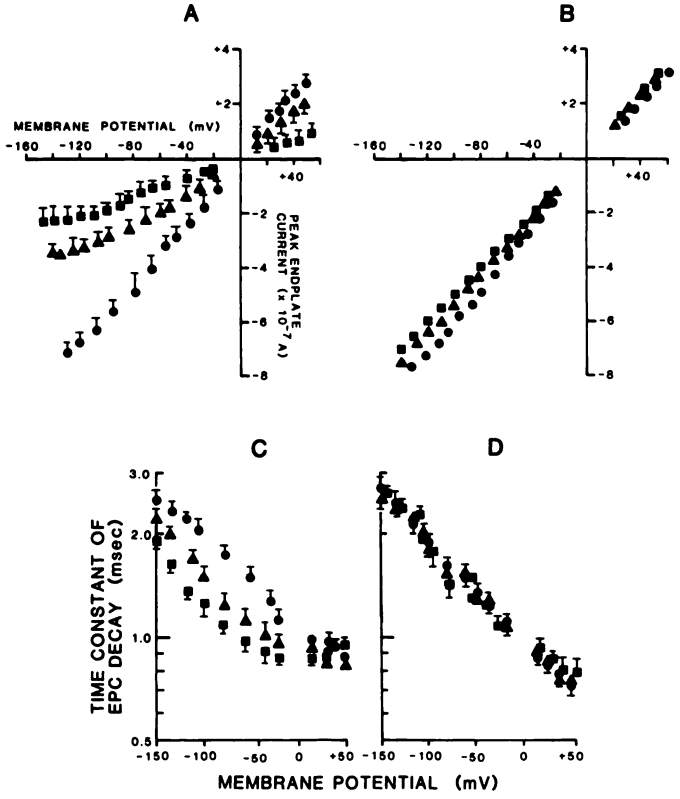

FIG. 8. *Current voltage relationships (A and B) and time constant of decay (C, D) of the EPC in the presence of piperocaine methiodide*

A and *C* show results obtained from recordings in control condition (●) and recordings made after 3 (▲) and 8 mins (■) of injection of drug from a pipette containing 0.01 M piperocaine methiodide (950 nA current passed through a pipette having a resistance of 25 MΩ). In *A* and *C*, the pipette was located at the center of the end plate region. *B* and *D* also show control condition (●) and recordings made after 3 (▲) and 9 (■) min of internal application of piperocaine methiodide, but at a distance greater than 250–350 μm from the center of the end plate region. In both cases, to assure that the pipette was in an intracellular position after the final record, a square pulse was applied to the pipette and the electronic potential was observed on the oscilloscope screen.

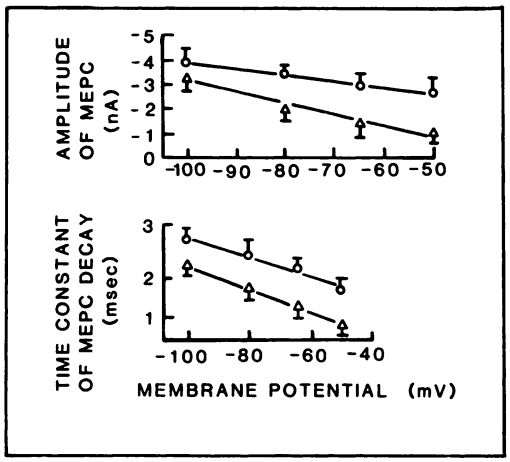

FIG. 9. *Effect of piperocaine methiodide on the spontaneous MEPC*

MEPC peak amplitude and τ in control (○) and in the presence of piperocaine methiodide internally applied (△) (900 nA, 2 min). Each *symbol* represents the mean and standard error of the mean of 8 to 17 MEPCs.

nonlinearity in the current-voltage relationship at negative membrane potentials (Fig. 8A). However, when piperocaine methiodide was applied internally but at a distance of 250–350 μm from the end plate region, no significant effects were seen on the peak amplitude and τ of the EPC (Fig. 8B and D) or MEPC. The effect of piperocaine methiodide on τ_{EPC}, τ_{MEPC}, and τ_{MEPC} spectra (Figs. 8–10) was more significant at less negative membrane potentials than at more negative membrane potentials (Fig. 8C). This constituted a marked departure from the shortening of τ_{EPC} and τ_{MEPC} which occurred at more negative potentials when the agent was applied extracellularly (compare Figs. 4 and 8). Piperocaine methiodide did not have a significant effect on the rise time of the EPC or MEPC.

Effect of N-methyl piperidine methiodide on the end plate current of the frog sartorious muscle. It was essential that the quaternary compounds be free of any of the

precursor tertiary amine. Since the actions of the tertiary amines could not be defined when they were applied inside the cell because of their lipophilic nature, it was important to determine whether or not PCP methiodide and piperocaine methiodide had any residual tertiary amine. Chemical analysis of synthetic PCP methiodide showed a significant contaminant (10–15%), a quaternary product identified as *N*-methyl piperidine methiodide, and no traces of any tertiary compounds such as PCP were found. As a control, the effect of intracellular application of *N*-methyl piperidine methiodide was investigated with micropipettes containing 0.01 M concentrations of the agent. Measurement of the current-voltage relationship for EPC peak amplitude and τ_{EPC} clearly shows that *N*-methyl piperocaine methiodide (50 μM) did not affect the ionic channel of the ACh receptor when applied to either outside or inside compartments of the cell at the level of the junctional membrane.

Effect of external and internal application of quaternary atropine methyl bromide to the muscle membrane at the junctional region. Atropine methyl bromide applied extracellularly has an effect similar to that seen with atropine sulfate (2). Atropine methyl bromide (40 μM) reduced the peak EPC amplitude, especially at hyperpolarized potential, thus resulting in a nonlinear-voltage pattern (Fig. 11A). The agent caused a 52% shortening in τ_{EPC} value obtained at −90 mV, and the voltage sensitivity of the EPC decay, expressed as the slope of the plot of logarithm of the τ versus membrane potential,

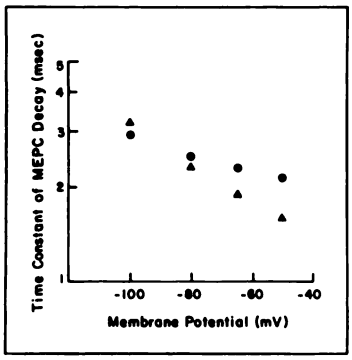

FIG. 10. *Time constant of decay of the MEPC* (τ) *obtained from MEPC spectra at different membrane potentials, in control* (●) *and after injection of piperocaine methiodide* (▲)

Spectra were obtained for two to seven MEPCs. Note the agreement with Fig. 9, where the data were obtained by a different method.

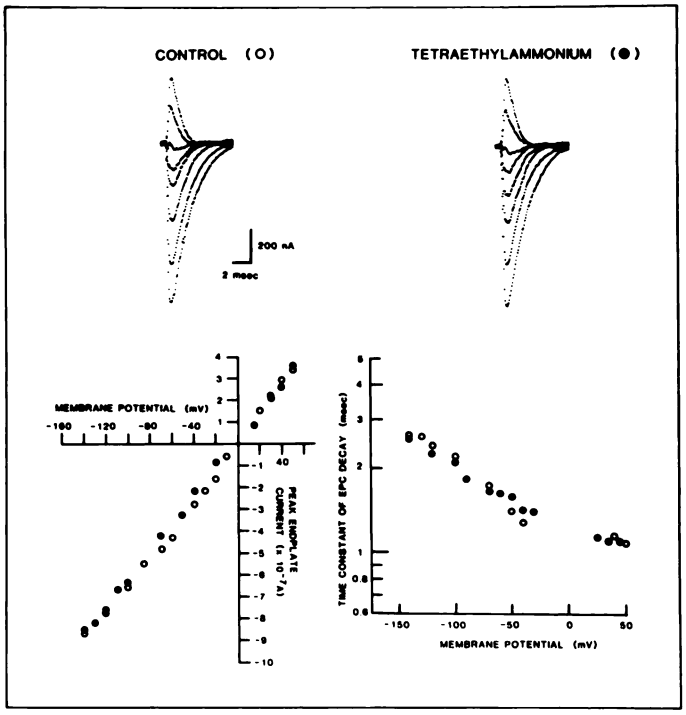

FIG. 12. *Effect of intracellular application of TEA on the EPC*

Families of EPCs recorded at membrane potentials from −140 to +50 mV under control conditions (*upper left family*) and after the internal injection of TEA (950 nA, 3.30 min) (*upper right family*). Both families were recorded from a single frog sartorius surface fiber. *Lower panels* show the current-voltage relationship of the EPC peak amplitude, and semilogarithmic plots illustrate the relationships between the time constant of EPC decay and membrane potential recorded in control (○) and after injection of the agent (●).

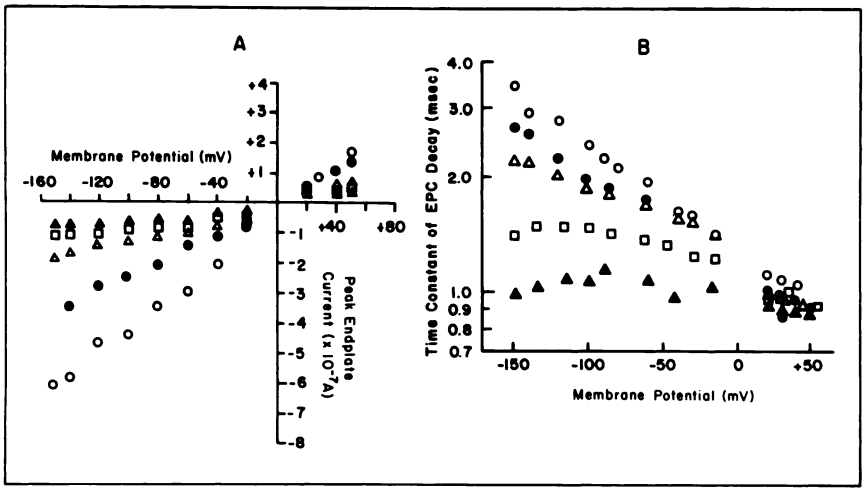

FIG. 11. *The effect of atropine methyl bromide on the current-voltage relationship of the EPC peak amplitude* (*A*) *and a plot showing the decay time constant as function of membrane potential* (*B*) *in control* (○) *and after the addition of various concentrations of the agent: 5 μM* (●), *10 μM* (△), *30 μM* (□), *and 40 μM* (▲)

Each *point* represents mean values from 6 to 10 end plates.

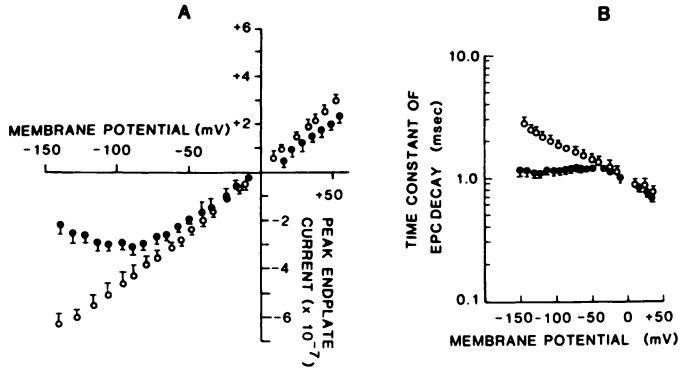

FIG. 13. *The effect of microiontophoretic application of TEA to the external surface of the end plate region*

Peak EPC amplitude (*A*) and τ_{EPC} (*B*) are plotted against membrane potential. Control (○) and in the presence of TEA externally applied (●). The pipette was filled with 1 M concentration of the agent and had a resistance of 5 MΩ. The microiontophoretic pulse was 1000 nA, 3.3 min.

was markedly reduced (Fig. 11B). When atropine methyl bromide was applied intracellularly by passing 800 nA of current for 2 min, no significant effect was recorded on EPC and MEPC; yet, as observed previously with atropine, the action potential was also prolonged. This indicates that significant amounts of the agent had reached the internal membrane. The voltage dependence of both the peak amplitude and τ of the EPC or MEPC remained literally identical with the control condition.

Effect of external and internal application of TEA to the muscle membrane at the junctional region. External application of TEA at concentrations 500 μM reduced peak amplitude and τ to about 36 and 48% at −150 mV and had little effect at +50 mV. The results were identical with those reported previously (3). In sharp contrast to PCP methiodide, intracellular injection of TEA from micropipettes containing the agents at a concentration of 2 M did not effect either the τ_{ECP} or EPC peak amplitude (Fig. 12). However, when the same micropipette was positioned extracellularly at the end plate region, and the same current passed (1000 nA for 3.3 min) as that used for the intracellular region, an effect on the amplitude and τ of the EPC similar to that of TEA applied in the bath was recorded. The lack of effect of TEA applied inside the cell at the region of the ionic channel of the ACh receptor indicates absence of active binding site at this region of the channel (Fig. 13). It should be mentioned that the successful effect of TEA applied externally via a micropipette is markedly dependent upon the visual light microscopic control of the micropipette location at the focal zone of the junctional region.

DISCUSSION

The present investigation has shown that PCP methiodide and piperocaine methiodide are able to affect significantly the voltage sensitivity of the ionic channel of the ACh receptor, regardless of whether they are applied outside the cell in the bath or inside the cell via microiontophoresis. The intracellular effects of these quaternary compounds on the ACh ion channel were easily seen when a sufficient quantity of the agent was microiontophoretically released into the cell (see Figs. 3, 7, and 8). The detection of binding sites for agents on the intracellular surface of the ACh receptor ionic channel complex confirms earlier findings of this laboratory (5, 11). The actions of PCP methiodide and piperocaine methiodide were seen to occur on the EPCs elicited by nerve stimulation and on the spontaneous MEPCs (see Figs. 3 and 7–10).

The effects of intracellularly applied PCP methiodide and piperocaine methiodide differ from those seen when the agent was applied extracellularly. For example, applied externally, the quaternary amines were most effective in altering the τ-membrane potential relationship and inducing a significant curvature in the current-voltage relationship at more hyperpolarized potentials, whereas in contrast, the internally applied agents had their greatest effect on the τ at less negative membrane potentials (see Figs. 6 and 8). The association and dissociation between the agent and its binding site located intracellularly depends on the membrane potential. This fact can be explained by suggesting that when the membrane is less polarized, a small fraction of ions (inward current) does not have enough energy to displace a PCP methiodide or piperocaine methiodide molecule from its binding site, but they gain energy moving through the channel when the voltage becomes more negative (increasing driving force), and thus dissociation is accelerated. This phenomenon has also been seen by other investigators studying sodium channel in nerve (18) and potassium channels in squid giant axons (8).

There are three significant factors related to the intracellular reaction of these agents which should be considered in the study. These relate to the following. (a) A leakage of the agent from the inside to the outside surface of the cell could possibly exist. However, in this situation, the voltage-dependence of τ must be similar regardless of whether the agent was internally or externally applied, and this was not the case (Figs. 3 and 9). Furthermore, if the drug leaks from the intracellular compartment of the muscle fiber, the leak rate of the positively charged agents should be larger at positive membrane potential; however, as shown in Figs. 6D and 8C, the effects were minimal when compared with the effects at −60 mV. In addition, while the EPCs or MEPCs were being recorded under internal injections of the agents, the bathing Ringer's solution was flowing at a fast rate (5 ml/min) over the preparation; thus, any residual effect of the agents which conceivably could leak outside would be minimal. This precaution was more of an assurance because PCP methiodide is readily washed out from the external media once the drug is replaced by Ringer's solution. It should be mentioned that the internally applied drug continued to affect both EPC and action potential for over 30 min (see Fig. 5). (b) Chemical contamination, i.e., the presence of small amounts of the lipophilic tertiary amine as a contaminant of the quaternary amine, should be considered. However, both PCP methiodide and piperocaine methiodide did not have detectable tertiary amine contaminants. Although PCP methiodide contained a quaternary by-product identified as *N*-methyl piperidine methiodide, this by-product had no effects either inside or outside the cell. An important

control with regard to technical aspects of the injection of the agents was the studies with TEA and atropine methyl bromide. The negative results with the latter agents indicate that the injury of impalement followed by release of a quaternary compound or by passing current do not in themselves affect the EPCs. Micropipettes with PCP methiodide and piperocaine methiodide had a concentration of 0.01 and 0.001 M of the agents, respectively, whereas the micropipettes with TEA and atropine methyl bromide had a 2 M concentration. In addition, although TEA and atropine methyl bromide were applied with a longer releasing time of the order of 3 min and with greater current (about 1 μA), there was no detectable alteration in either MEPCs or EPCs. The absence of effects indicates that the effects of PCP methiodide or piperocaine methiodide applied inside the cells reflect interaction of these compounds with specific sites, and not an artifact of technique. (c) A factor which may contribute to the less intensive effect of the agents is related to a much lesser extension of the ionic channel into the intracellular regions (15 Å versus 55 Å, extracellular) (21, 22). Conceivably, the closer the channel is to the bilayer membrane, the narrower the channel may be. Thus, under this condition one would expect that the outer mouth of the extracellular portion of the channel may have a large lumen and bigger area in contrast to the inner mouth of the intracellular portion of the channel. This could make fewer sites available for agents inside than outside the cell. In addition, the fact that drugs such as PCP methiodide and piperocaine methiodide sense remarkably little of the membrane surface, and other drugs such as HTX (5) and QX-222 (20) sense the membrane potential via sites located deep down into the channel, raise the possibility that the narrowed section of the channel (6–7 A) is located in very close proximity to the intracellular portion of the ionic channel macromolecule (23).

Since PCP methiodide and piperocaine methiodide are able to affect the ionic channel of the ACh receptor from both outside and inside the cell, it would appear that there are binding sites for such agents located on both the inner and outer aspect of the ionic channel. Another important feature appears to be that drugs such as atropine methyl bromide and TEA, when applied extracellularly, react with the ionic channel in a voltage-dependent manner; however, these agents have no effect on the ionic channel when microinjected inside the cell. In contrast, agents such as PCP methiodide and piperocaine methiodide, which react with the ionic channel in a voltage-dependent manner when applied extracellularly, also reacted with the intracellular region of the ionic channel. Since the PCP methiodide and piperocaine methiodide molecules sense the membrane potential by 6%, it is possible that most of these agents react on the surface or at the boundaries of the channel, affecting its conformational state such that acceleration of time constant of decay and depression of peak amplitude can be seen. Such a surface location is contrary to the site of action of the quaternary amine, QX-222, which appears to be located at a depth equal to about 78% of the length of the channel. There is evidence which could indicate that certain quaternary local anesthetics act from inside the ACh receptor at the frog neuromuscular junction. Indeed, Katz and Miledi (13) found a small inhibition with QX-222 internally applied. Bath-applied QX-222 alters the appearance of the EPC, which in the presence of the drug decays as the sum of two exponential functions (24). This suggests that the kinetics of interaction of QX-222 with ACh ion channel complex are different from those for PCP methiodide and piperocaine methiodide. With the latter compounds, EPC or MEPC showed a single exponential decay regardless of whether the drug was applied to the outside or inside of the membrane. If one assumes that the height of the energy barrier of the ion selectivity of the ACh ion channel is located midway through the electric constant field (25), the neighboring side of the barrier may have sites with selectivity toward certain charged molecules, such as PCP methiodide and piperocaine methiodide. It has been suggested (26) that the voltage dependence of single-channel conductance seen with different cations may be the result of negative dipole charges which could serve as binding sites within the channel. In addition, as was mentioned previously, the physical insertion of the ACh receptor ionic channel complex into the membrane with 15 Å facing the intracellular aspect of the membrane suggests that the narrow portion of the channel is indeed closer to the intracellular region near to the membrane proper. It is in this area that the selectivity filter might be located. Since the molecule of TEA is small enough to reach deep into the channel from intracellular aspects while PCP methiodide and piperocaine methiodide are not, one has to assume that it is binding sites on the surface of the channel that are absent for TEA. In fact, PCP methiodide or piperocaine methiodide after internal application to the channel appear to move to the channel and interact with it more strongly than other molecules such as TEA, atropine methyl bromide, or *d*-tubocurarine.[5] Our negative results with intracellular application of *d*-tubocurarine are similar to those described by Magazanik and Vyskocil (27) and Katz and Miledi (13). Agonists such as ACh and carbamylcholine, which are quaternary amines, and anatoxin-a, a secondary amine (28), were found to have no effect when applied inside the cell.[6]

These findings suggest that molecules such as PCP methiodide and piperocaine methiodide can affect the channel at the boundaries near the surfaces. Externally, small cations such as TEA, which sense the membrane potential more than PCP methiodide, or piperocaine methiodide have effects, but are devoid of any action when applied inside the cell. Perhaps a large molecule is necessary to react with multiple sites located around the periphery of the channel opening. These results further indicate that the interaction of tertiary amines and certain quaternary amines with such sites is affected by membrane potential. From the outside, the more negative the membrane potential, the greater the access of a positively charged species, whereas drug application from the inside would tend to dissociate the agent faster from its binding sites. Since tertiary amines upon equilibration

[5] L. G. Aguayo, B. Pazhenchevsky, J. W. Daly, and E. X. Albuquerque, unpublished results.

[6] C. E. Spivak and E. X. Albuquerque, unpublished results.

reach both outside and internal sites most likely equally, the resultant decay time constant may be an average due to the drug action at these two opposite sites.

In conclusion, the present investigation suggests that the ionic channel of the ACh receptor has reactive sites for quaternary amines, and presumably the corresponding protonated tertiary amines both outside and inside the cell. Further studies will be required to assess the relative importance of extracellular and intracellular sites on the acetylcholine receptor-ionic channel complex to the action of other local anesthetics and the histrionicotoxins.

ACKNOWLEDGMENTS

The authors are grateful to Professor David Triggle for the gift of PCP methiodide. We are most indebted to Dr. Harel Weinstein and Dr. Saul Mayaani for the most valuable suggestions for and criticisms of the manuscript. We are also grateful to Dr. Daniel Weinreich for the technical suggestions regarding intracellular release of drugs. The expert computer analysis and general technical assistance of Ms. Mabel Zelle are greatly appreciated.

REFERENCES

1. Adams, P. R. Voltage jump analysis of procaine action at frog endplate. *J. Physiol.* (*Lond.*) **268**:291–318 (1977).
2. Adler, M., and E. X. Albuquerque. An analysis of the action of atropine and scopolamine on the end-plate current of frog sartorius muscle. *J. Pharmacol. Exp. Ther.* **196**:360–372 (1976).
3. Adler, M., A. C. Oliveira, E. X. Albuquerque, N. A. Mansour, and A. T. Eldefrawi. Reaction of tetraethylammonium with the open and closed conformations of the acetylcholine receptor ionic channel complex. *J. Gen. Physiol.* **74**:129–152 (1979).
4. Albuquerque, E. X., M.-C. Tsai, R. S. Aronstam, A. T. Eldefrawi, and M. E. Eldefrawi. Sites of action of phencyclidine. II. Interaction with the ionic channel of the nicotinic receptor. *Mol. Pharmacol.* **18**:167–178 (1980).
5. Albuquerque, E. X., M. Adler, C. E. Spivak, and L. G. Aguayo. Mechanism of nicotinic channel activation and blockade. *Ann. N. Y. Acad. Sci.* **358**:204–238 (1980).
6. Albuquerque, E. X., E. A. Barnard, T. H. Chiu, A. J. Lapa, J. O. Dolly, S.-E. Jansson, J. Daly., and B. Witkop. Acetylcholine receptor and ion conductance modulator sites at the murine neuromuscular junction: evidence from specific toxin reactions. *Proc. Natl. Acad. Sci. U. S. A.* **70**:949–953 (1973).
7. Shanes, A. M. Electrochemical aspects of physiological and pharmacological action in excitable cells. Part II. The action potential and excitation. *Pharmacol. Rev.* **10**:165–273 (1958).
8. Armstrong, C. M. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. Gen. Physiol.* **58**:413–437 (1971).
9. Lapa, A. J., E. X. Albuquerque, J. M. Sarvey, J. Daly, and B. Witkop. Effect of histrionicotoxin on the chemosensitive and electrical properties of skeletal muscle. *Exp. Neurol.* **47**:558–580 (1975).
10. Albuquerque, E. X., M.-C. Tsai, R. S. Aronstam, B. Witkop, A. T. Eldefrawi, and M. E. Eldefrawi. Phencyclidine interactions with the ionic channel of the acetylcholine receptor and electrogenic membrane. *Proc. Natl. Acad. Sci. U. S. A.* **77**:1224–1228 (1980).
11. Aguayo, L. G., and E. X. Albuquerque. Internal and external sites of action of quaternary compounds at the nicotinic acetylcholine receptor-channel complex. *Soc. Neurosci. Abstr.* **6**:836 (1980).
12. Horn, R., M. S. Brodwick, and W. D. Dickey. Asymmetry of the acetylcholine channel revealed by quaternary anesthetics. *Science* (*Wash. D. C.*) **210**:205–207 (1980).
13. Katz, B., and R. Miledi. Blockade of endplate response by intracellular application of procaine, in *Ontogenesis and Functional Mechanisms of Peripheral Synapses* (J. Taxi, ed.). Elsevier/North Holland Biomedical Press, Amsterdam, 171–178 (1980).
14. Albuquerque, E. X., J. E. Rash, R. F. Mayer, and J. R. Satterfield. An electrophysiological and morphological study of the neuromuscular junction in patients with myasthenia gravis. *Exp. Neurol.* **51**:536–563 (1976).
15. Tiedt, T., E. X. Albuquerque, N. Bakry, M. E. Eldefrawi, and A. T. Eldefrawi. Voltage- and time-dependent action of piperocaine on the ion channel of the acetylcholine receptor. *Mol. Pharmacol.* **16**:909–921 (1979).
16. Gage, P. W., and R. N. McBurney. Effects of membrane potential, temperature and neostigmine on the conductance change caused by a quantum of acetylcholine at the toad neuromuscular junction. *J. Physiol.* (*Lond.*) **244**:385–407 (1975).
17. Katz, B., and R. Miledi. The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.* (*Lond.*) **244**:665–699 (1972).
18. Woodhull, A. M. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* **61**:687–708 (1973).
19. Adler, M., E. X. Albuquerque, and F. J. Lebeda. Kinetic analysis of endplate currents altered by atropine and scopolamine. *Mol. Pharmacol.* **14**:514–529 (1978).
20. Neher, E., and J. H. Steinbach. Local anesthetics transiently block currents through single acetylcholine receptor channels. *J. Physiol.* (*Lond.*) **277**:153–176 (1978).
21. Klymkowsky, M., and R. Stroud. Immunospecific identification and three-dimensional structure of a membrane bound acetylcholine receptor from torpedo californica. *J. Mol. Biol.* **128**:319–334 (1979).
22. Ross, M. J., M. W. Klymkowsky, D. A. Agard, and R. M. Stroud. Structural studies of a membrane-bound acetylcholine receptor from torpedo californica. *J. Mol. Biol.* **116**:635–659 (1977).
23. Dwyer, T. M., D. J. Adams, and B. Hille. The permeability of the endplate channel to organic cations in frog muscle. *J. Gen. Physiol.* **75**:469–492 (1980).
24. Ruff, R. L. A quantitative analysis of local anaesthetics alteration of miniature endplate currents and endplate current fluctuations. *J. Physiol.* (*Lond.*) **264**:89–124 (1977).
25. Steinbach, J. H., and C. F. Stevens. Neuromuscular transmission, in *Frog Neurobiology* (R. Llinas and W. Precht, eds.). Springer-Verlag, New York, 33–92 (1976).
26. Gage, P. W., and D. Van Helden. Effects of permeant monovalent cations on endplate channels. *J. Physiol.* (*Lond.*) **288**:509–528 (1979).
27. Magazanik, L. G., and F. Vyskocil. The loci of α-bungarotoxin action on the muscle postjunctional membrane. *Brain Res.* **48**:420–423 (1972).
28. Spivak, C. E., B. Witkop, and E. X. Albuquerque. Anatoxin-a: a novel, potent agonist at the nicotinic receptor. *Mol. Pharmacol.* **18**:384–394 (1980).

Send reprint requests to: Dr. Edson X. Albuquerque, Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Md. 21201.