Rates and Equilibria at the Acetylcholine Receptor of *Electrophorus* Electroplaques

A Study of Neurally Evoked Postsynaptic Currents and of Voltage-Jump Relaxations

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ABSTRACT Kinetic measurements are employed to reconstruct the steady-state activation of acetylcholine [ACh] receptor channels in *Electrophorus* electroplaques. Neurally evoked postsynaptic currents (PSCs) decay exponentially; at 15°C the rate constant, α, equals 1.2 ms^{-1} at 0 mV and decreases e-fold for every 86 mV as the membrane voltage is made more negative. Voltage-jump relaxations have been measured with bath-applied ACh, decamethonium, carbachol, or suberyldicholine. We interpret the reciprocal relaxation time $1/\tau$ as the sum of the rate constant α for channel closing and a first-order rate constant for channel opening. Where measurable, the opening rate increases linearly with [agonist] and does not vary with voltage. The voltage sensitivity of small steady-state conductances (e-fold for 86 mV) equals that of the closing rate α, confirming that the opening rate has little or no additional voltage sensitivity. Exposure to α-bungarotoxin irreversibly decreases the agonist-induced conductance but does not affect the relaxation kinetics. Tubocurarine reversibly reduces both the conductance and the opening rate. In the simultaneous presence of two agonist species, voltage-jump relaxations have at least two exponential components. The data are fit by a model in which (*a*) the channel opens as the receptor binds the second in a sequence of two agonist molecules, with a forward rate constant to 10^7 to 2×10^8 M^{-1} s^{-1}; and (*b*) the channel then closes as either agonist molecule dissociates, with a voltage-dependent rate constant of 10^2 to 3×10^3 s^{-1}.

INTRODUCTION

This paper concerns the rate-limiting steps in the gating of ion channels by nicotinic acetylcholine (ACh) receptors. In principle these steps could be studied most directly by monitoring membrane conductance as it responds to rapid jumps upward and downward in agonist concentration. Such "agonist concentration-jumps" are presently obtained under special circumstances. For instance, *trans*-bis-Q, an agonist, can be generated from the relatively inactive *cis* isomer by light flashes (Lester and Chang, 1977). Another agonist concentration-jump occurs after the presynaptic nerve terminal releases quanta of acetylcho-

line at amphibian endplates. Receptor channels open during the first 0.3 ms; transmitter then seems to vanish from the synaptic cleft before an appreciable fraction of channels have closed again. Therefore the declining phase of the neurally evoked postsynaptic current (PSC) reflects the "relaxation" kinetics of the channel population after a nearly instantaneous removal of ACh (Magleby and Stevens, 1972*a*,*b*; Kordas, 1972*a*,*b*; Gage and McBurney, 1975). A similar situation occurs at *Electrophorus* nerve-electroplaque synapses (Sheridan and Lester, 1975*a*); we have used this fact in the present study.

There is, moreover, a relaxation measurement which seems useful for all agonists. The steady-state agonist-induced conductance depends on the membrane potential as well as on the concentration of agonist (Ruiz-Manresa and Grundfest, 1971; Rang, 1973; Dionne and Stevens, 1975; Lester et al., 1975; Adams, 1976). It is thus possible to study the agonist-induced conductance in a fashion analogous to the voltage-clamp investigations of axons. In brief, we have allowed the electroplaque membrane to equilibrate with a steady concentration of agonist. The transmembrane voltage is then jumped from one value to another under feedback control, and the agonist-induced conductance subsequently relaxes to a new equilibrium value, on a millisecond time scale. In its detailed time course and amplitude this relaxation reflects the rate processes underlying cholinergic excitation (Eigen and DeMaeyer, 1974).

From analyses of fluctuations and of discrete components in amphibian endplate currents, it is known that single ion channels, the molecular basis of the agonist-induced conductance, have a conductance γ of roughly 3×10^{-11} mho and a lifetime, depending on several factors, of 1–40 ms (Katz and Miledi, 1972; Anderson and Stevens, 1973; Neher and Sakmann, 1976*a*). This channel lifetime is also measured by voltage-jump relaxations at low agonist concentrations (Adams, 1975*a*; Neher and Sakmann, 1975, 1976*b*) and, for the case of ACh, by the declining phase of the PSC (Magleby and Stevens, 1972*a*,*b*; Anderson and Stevens, 1973). The present experiments show that receptor channels in *Electrophorus* electroplaques have a similar lifetime and that the lifetime is similarly influenced by the nature of the agonist and by the membrane potential. We also extend the molecular description by measuring the *frequency* of elementary events, or the rate constant for opening channels, as influenced by the same factors and by the concentration of agonist.

As first noted by Katz and Thesleff (1957) at frog endplates, small agonist-induced conductances increase more than linearly with the concentration of agonist. The same is true at *Electrophorus* nerve-electroplaque junctions (Lester et al., 1975). The present relaxation measurements provide tentative reasons for selecting one of several molecular models which have been proposed to explain this interesting behavior (Changeux et al., 1967; Karlin, 1967*a*; Changeux and Podleski, 1968; Colquhoun, 1973). Some of the data have already been published in briefer form (Sheridan and Lester, 1975*a*,*b*; Lester and Sheridan, 1976).

MATERIALS AND METHODS

The experiments employ single, isolated electroplaques from the organ of Sachs of *Electrophorus electricus*. Animals 1–1.5 m in length were purchased from Paramount Aquarium or its successor, World-Wide Scientific Animals, Ardsley, N. Y. They were

maintained in individual tanks (200 liter) in distilled water with 0.5% seawater at 25°C. A series of experiments lasted 5–8 days. Fresh slices several centimeters thick were cut from the fish daily; the wound was then cauterized.

Solutions

We substituted 10 mM HEPES buffer for the previously used phosphate buffer (Lester et al., 1975); this procedure seemed to increase viability of electroplaques. Suberyldicholine (Sub) was a gift of J. Heesemann; α-bungarotoxin was a gift of M. A. Raftery. Methanesulfonyl fluoride (MSF) was obtained from Eastman Kodak Corp., Rochester, N. Y.; other organic chemicals were from Sigma Chemical Co., St. Louis, Mo., and from K & K Laboratories, Plainview, N. Y.

Electrical Arrangements

The electroplaque was held so that part of its innervated face was exposed to a pool of Ringer solution (pool A) through a 1 × 3-mm window in a thin Mylar sheet. The entire noninnervated face was exposed to another pool, termed B.

As in our previous studies (Lester et al., 1975; Sheridan and Lester, 1975*a*), current was applied to the membrane only during the actual clamping episodes. The output of the clamping amplifier was led through an electronic switch to the plate used to apply current in pool B. The switch was closed a few milliseconds before the clamping episode, which lasted 15–170 ms, and opened immediately afterwards. The plate in pool A was connected to a virtual ground circuit for measuring currents.

The clamping circuit provided for two alternative means of measuring the potential across the innervated membrane. In the "transmembrane" method, two microelectrodes straddled this membrane and their signals were compensated for series resistance (Nakamura et al., 1965; Lester et al., 1975). The second ("transcellular") method exploited the stability, low noise, and small time constant of large-tipped extracellular pipettes. In brief, two such electrodes sensed the IZ drop across the whole cell; from this value the circuit subtracted IZ drops across series resistances and across the noninnervated face. The zero-current potential of the innervated membrane was measured independently, during zero current flow, by an intracellular electrode. This value was added to the corrected IZ drop, the result being potential across the innervated membrane. The intracellular electrode was included primarily to avoid errors in the voltage measurements due to the slow shift of zero-current potentials during prolonged exposure to agonist (Lester et al., 1975). This effect arises because internal K^+ is replaced by Na^+ (Karlin, 1967*b*; Lester, 1977). The shift proceeds at a maximum rate of about 1 mV/s, too slow to distort measurements during an individual clamping episode.

In the transcellular method the extracellular electrodes, 1 and 3, were filled with 1 M NaCl, had tip diameters of about 50 μm, and had resistances of about 200 kΩ. They were placed, respectively, close to the noninnervated face (in pool B) and the innervated face (pool A). These two electrodes were thus directly opposite each other, straddling the portion of the cell exposed through the window and about 200 μm apart. Electrode 2, a KCl-filled micropipette with a tip resistance of 5–10 MΩ, was inserted into the electroplaque from pool A. The difference, $V_1 - V_2$, was fed to a track-and-hold circuit controlled by the same logic signal as the electronic switch on the voltage clamp output. If externally applied current, I, is zero, $V_1 - V_2$ is the negative of the EMF across the noninnervated face. Between clamping episodes the electronic switch was open, I was zero, and the circuit tracked $V_1 - V_2$. Just before the start of an episode, the circuit was commanded to hold its present value, which we designate as $(V_1 - V_2)_0$. The electronic switch was then closed, completing the feedback loop and allowing a current I to flow

across the electroplaque. Analog circuits computed the membrane potential, V, for input to the feedback circuit:

$$V = V_1 - V_3 - IR_s - V_{ni} - (V_1 - V_2)_0.$$

Here R_s is the series resistance of the Ringer solution between electrodes 1 and 3. V_{ni} is the IZ drop across the impedance of the noninnervated face and was determined by forcing the current I through a resistance-capacitance model of the noninnervated face (Cohen et al., 1969); this correction was typically 20% as large as IR_s.

The transcellular method is valid if the zero-current potential of each face does not change during the clamping episode. Zero-current potentials were noted before and after clamping episodes and usually differed by less than 1 mV; thus this condition was fulfilled.

By comparison with the transmembrane method, the transcellular method's chief advantage is the faster clamping speed, allowing the membrane capacitance to be charged with a typical time constant of 30 μs. For responses slow enough to fall within the temporal resolution of the transmembrane method, the two procedures yielded identical results.

Voltage-clamp trials (e.g., Figs. 1 and 3) usually consisted of 8–16 episodes, lasting 15–170 ms each, controlled by a digital minicomputer. The computer furnished the voltage-clamp circuit with analog command signals for the membrane voltage, while at the same time accepting from that circuit analog signals proportional to the measured membrane potential and to the membrane current. The computer also furnished logic signals which controlled the electronic switch and the track-and-hold circuit.

Neurally Evoked Postsynaptic Currents (PSCs)

Clamping episodes were devised to produce PSCs uncomplicated by currents through electrically excitable channels in the innervated face. Each PSC episode started with a 10–15-ms clamp to +50 mV. This produced the usual Na currents which then inactivated. Delayed K currents do not occur in the electroplaque. A brief (<0.5 ms), strongly positive command pulse (+100 to +250 mV) was then given; the resulting current evoked an impulse in the presynaptic terminals. At the conclusion of this pulse, the membrane was then clamped to the desired voltage for monitoring the PSC. Only this final voltage varied among the episodes of a trial. For simplicity the traces of Fig. 1 start at the final voltage level.

In order to obtain the largest possible PSCs, we facilitated release by repeating episodes at a frequency of 3/s. PSC amplitude reached a steady state during an introductory series of 10 episodes which preceded each trial.

Measurement of Agonist-Induced Currents

Other excitable responses were suppressed pharmacologically and passive currents were subtracted. Solutions contained Ba^{+2} (3 mM) to maintain the inward rectifier in a voltage-insensitive, low-conductance state (Ruiz-Manresa et al., 1970) and where appropriate, tetrodotoxin ($\sim 10^{-6}$ M) to eliminate Na activation. At 15°C and below, the small transient outward current evoked by depolarization (Lester et al., 1975) usually disappeared entirely or appeared only at potentials more positive than those used in the present study. Thus in the absence of agonist, only ohmic leakage currents (Lester et al., 1975) and capacitative currents remained.

In some experiments the capacitative and ohmic currents were measured by separate "control" voltage-clamp trials without agonist and without presynaptic stimulation. With agonist present, or with presynaptic stimulation, the identical sequence of voltages was

applied. To determine agonist-induced currents, the control trials were subtracted from the "agonist" trials.

Some voltage-jump experiments (e.g., Fig. 3) employed a "single-trial" method based on the fact that the agonist-induced slope conductance vanishes at potentials more positive than about +30 mV (Lester et al., 1975). Even during steady exposure to agonist, if voltage jumps remain within the range of +30 mV to +100 mV, only passive currents are recorded. Clamping episodes were devised in which two jumps were measured, the first within this positive range and a second either to, from, or within the region of negative potentials where agonist-induced conductance was present. The computer scaled the passive currents by the appropriate factor, then subtracted them from the currents of the second jump. Results with this method were usually the same as those with the method of separate agonist and control trials. We prefer the single-trial method because it lent itself readily to on-line computation of agonist-induced currents and to on-line averaging of signals from multiple trials (2–16 were used); furthermore, the method was insensitive to small changes of passive conductance between agonist and control trials.

SODIUM CHANNEL GATING CURRENTS In the electroplaque as in other tissues with excitable sodium channels, gating currents appear as voltage-sensitive capacitative currents (Armstrong, 1975). Gating currents are isolated, not eliminated, by the single-trial method. Agonist-induced depolarization partially inactivates gating currents; therefore the method of separate trials yielded inverted gating currents. The gating currents were small (0.5–1 mA/cm^2) and declined with time constants at least 10 times shorter than those of the relaxations described in this paper (H. A. Lester and D. D. Koblin, unpublished results). Therefore, sodium channel gating currents usually caused little distortion in the relaxations.

Voltage-Clamp Fidelity

SERIES RESISTANCE COMPENSATION With the transcellular method we measured R_s by assuming that it causes the nearly instantaneous change of $V_1 - V_3$ in response to a step of current. Values ranged from 1 to 1.7 Ω-cm^2. For the usual reasons involving clamp stability it was rarely possible to compensate more than 80% of R_s, leaving 0.2–0.35 Ω-cm^2 of uncompensated series resistance. During (*a*) PSCs or (*b*) exposure to high agonist concentrations at high negative potential, the membrane's resistance often decreased to 5 Ω-cm^2. In these cases, the measured voltage-clamp currents were probably 4–7% too low.

POSSIBLE EDGE EFFECTS If the cell is not completely sealed around the edges of the window, agonist could reach areas underneath the seal, and currents flowing through the window would only partially clamp these edge regions. Because the agonist-induced conductance decreases with depolarization, the edge currents would be largest, relative to the agonist-induced currents through the window, when the window was clamped at positive potentials. This effect could contribute to the apparently nonlinear instantaneous I–V plot of the receptor channels (see Results) at positive potentials. However, such partial clamping of the edge regions would also distort sodium currents in the region of the spike threshold. Therefore in preliminary experiments we measured the instantaneous I–V curve for Na currents using the method of repolarizing "tails" (Hodgkin and Huxley, 1952). The relation was linear near the threshold potential. We therefore feel that edge currents introduced negligible distortion in these experiments. Nonetheless, in its next version the voltage-clamp apparatus will have provision for focal recording to avoid edge currents.

CLAMPING THE STALKS Synapses occur on stalklike protrusions of the innervated face. The stalks have an average radius of 5 μm and a maximum length of 30 μm; they increase the area of the innervated face by roughly a factor of 4 (unpublished light and electron microscopy by A. Van Harreveld, J. Trubatch, and H. A. Lester). In this study

the resting membrane's resistance was about 60 mmho/cm^2 of window area (close to that found by Lester et al., 1975); accounting for the area of the stalks, the conductance is of the order of 15 mmho/cm^2. (There are microvesicular tubules, roughly 200 nm in diameter, which further increase the membrane's area by several fold and apparently account for this relatively high conductance.) With a cytoplasmic resistivity of 63 Ω-cm, these figures imply that the stalks have a DC space constant of 160 μm. The membrane time constant was about 200 μs. If one assumes that the stalks act like terminated cables it may be shown that, after a voltage step at the base of a stalk, the tip of the largest stalk is charged to more than 90% of this voltage within 20 μs (Waltman, 1966). Thus the stalks were well clamped in these experiments.

Conduct of the Experiments

SOLUTION CHANGES Usually, 10 ml of a new solution containing agonist was flushed through pool A (volume 0.8 ml) in 20 s by use of a push-pull syringe system. During and after the solution change, abbreviated voltage-clamp trials were given every 15–30 s. Agonist-induced conductances change less rapidly than do concentrations in the bulk solution, presumably because of connective tissue barriers covering the innervated face (Lester et al., 1975). The conductance reached a steady state after 0.5–5 min. This time decreased with increasing agonist concentrations and varied over a threefold range among cells tested at the same agonist concentration. Desensitization proceeds more slowly at and below 15°C than at room temperature (Magazanik and Vyskocil, 1975): for agonist concentrations used in the present study we found that conductances remained constant for at least 30 s before any desensitization was noted. This time sufficed for one or more complete voltage-clamp trials. Usually, pool A was then washed with agonist-free Ringer. "Cumulative" applications were used with low concentrations of agonist or when the response was kept small with *d*TC (see Fig. 6).

INACTIVATING THE ESTERASE Certain technical difficulties limit our ability to compare, on the same cell, neurally evoked PSCs and voltage-jump relaxations with ACh. The former experiment yields most useful information when the ACh pulse is kept brief by functional acetylcholinesterase, while the latter requires inactivation of the esterase for precise knowledge of ACh concentrations near the receptors. Prostigmine and eserine, two reversible cholinesterase inhibitors, seem to have additional direct effects upon the acetylcholine receptor (see Results). We have therefore relied upon the irreversible inhibitor MSF which need not be present in the bath during the relaxation measurements. In most experiments the PSCs were measured on the fresh cell, which was then treated with MSF (10 mM) for 1 h at 15°C. After several washes, voltage-jump trials were then taken with bath application of ACh. Responses to Sub were also larger after MSF treatment; there was no systematic effect on carbachol (Carb) or decamethonium (Deca) responses, so MSF treatment was usually omitted for experiments with the latter two drugs.

Systematic changes often occur in rates and equilibria after several exposures to agonist (D. D. Koblin and H. A. Lester, manuscript in preparation), and it was therefore disappointing to lack control data for PSC currents with active esterase after the bath application of ACh. As a partial check we have used the fact that exposure to *d*-tubocurarine (*d*TC) is expected to circumvent effects of buffered diffusion, allowing the ACh concentration to approximate a brief pulse even in the absence of esterase activity (Katz and Miledi, 1973; Magleby and Terrar, 1975). PSCs were therefore measured in *d*TC (1–4 μM) at the conclusion of some voltage-jump series. PSC amplitudes decreased by 3–13-fold; decay time constants were smaller than in the absence of *d*TC (on the average, by 60%) and agreed within 20% with the PSC data on the fresh cell. This finding suggests that no major changes in receptor kinetics occurred during the experimental series.

Computer Analysis of Agonist-Induced Currents

At all stages in analysis of the digitized currents, data (for instance those of Fig. 3) were displayed on an X-Y scope for inspection. Single exponentials were fit to the PSC decays and voltage-jump relaxations. As a first step, agonist-induced currents (Fig. 1*a* or 3*c*) were smoothed with a single- or double-pole recursive filter (time constant, 100 μs). Next, the final 10–20% of each trace was taken as the equilibrium (infinite time) value; this portion was checked for zero slope and displayed. The equilibrium value was then subtracted from each point in the trace. Generally, the initial 100–300 μs after a voltage-jump was ignored to avoid contamination by gating currents; but the results were not detectably sensitive to this starting point. Traces were usually truncated when the subtracted currents decreased to $0.3\sqrt{n}\%$ or $0.5\sqrt{n}\%$ of full scale, where n is the number of episodes averaged for each relaxation. The semilogarithmic plots of these data were usually straight (Figs. 1*b* and 3*d*); thus a linear least-squares fit to such plots gave the relaxation time constant, and extrapolation to the time of the jump gave the relaxation amplitude. For these cells the exact point of truncation had no effect on the analysis. A minority of episodes gave nonlinear plots; in such cases traces were truncated at a higher level until only linear portions remained.

The entire analysis was evaluated with exponential waveforms of varying amplitude generated by an RC circuit. The computed time constants did not vary detectably (<2%) with amplitude; computed and actual relaxation amplitudes agreed to within 5%.

RESULTS

Neurally Evoked Postsynaptic Currents (PSCs)

In regard to their kinetics and voltage sensitivity, PSCs in *Electrophorus* electroplaques strongly resemble the endplate currents of frogs and toads (Magleby and Stevens, 1972*a*,*b*; Kordas, 1972*a*,*b*; Gage and McBurney, 1975). Postsynaptic currents decay with a single exponential time constant (Fig. 1) which we term $1/\alpha$ for consistency with the terminology of Magleby and Stevens (1972*a*,*b*), Anderson and Stevens (1973), and Dionne and Stevens (1975). The rate α depends, in turn, exponentially upon membrane potential (Sheridan and Lester, 1975*a*), so that

$$\alpha = \alpha_0 e^{V/V_1}$$

At 15°C, α_0 equals 1.23 ± 0.12 ms^{-1} (mean ± SEM, seven cells) and V_1 equals 86 ± 6 mV (mean ± SEM, seven cells).

EFFECTS OF CHOLINESTERASE INHIBITORS Prostigmine bromide and MSF effected a clear lengthening of the PSC; the latter drug acts irreversibly on a time scale of several hours. The decay phase remained exponential. For both inhibitors, α_0 decreased by an average of 60% and continued to depend exponentially on membrane potential; V_1 changed by <10%. Similar effects with prostigmine, viz., a decrease in α_0 but no change in V_1, have been observed at endplates (Magleby and Stevens, 1972*a*, Gage and McBurney, 1975).

CURRENT-VOLTAGE RELATIONS AT THE PEAK OF THE PSC The relationship is approximately linear at higher negative potentials, but levels off to show a lower slope conductance at potentials more positive than about −30 mV (Figs. 1*c* and 2). Outward currents were quite small and variable among cells; we have seen actual reversal of the PSC in only 40% of all electroplaques studied (for example, Fig. 2). Where measurable at 15°C the reversal potential averaged +8 ± 6 mV

(mean ± SD, six cells), in reasonable agreement with the value of +2 ± 6 mV found recently by Lassignal and Martin (1977).

POSSIBLE REASONS FOR THE SMALL AND VARIABLE OUTWARD PSCS This situation has prevented us from extending our kinetic studies to positive potentials. Although we do not completely understand its causes, we touch briefly on several possibilities.

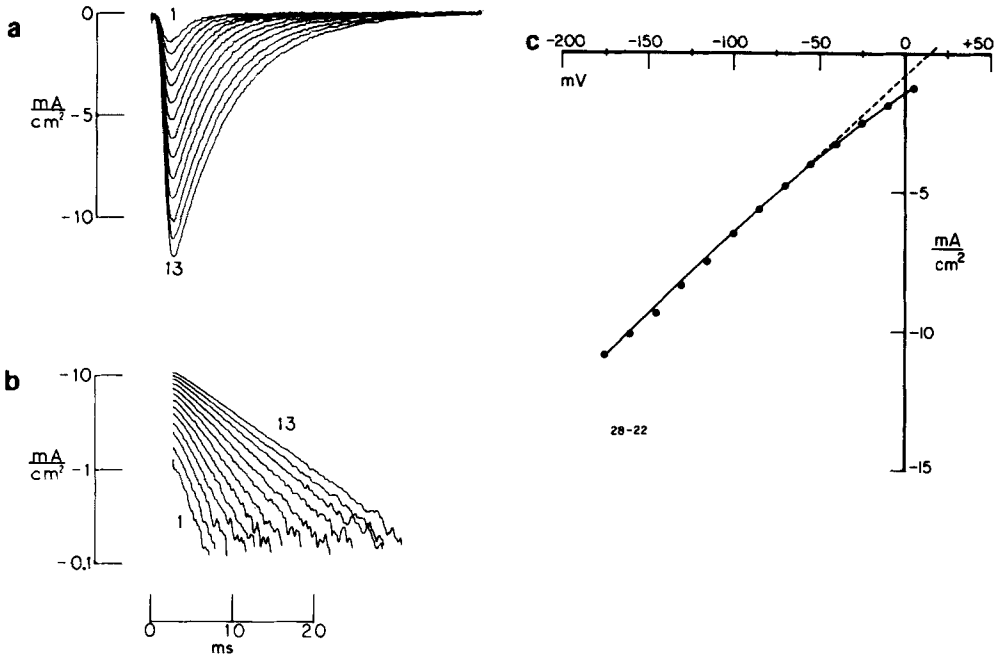

FIGURE 1. (*a*) Superimposed PSCs at membrane potentials from +5 mV (no. 1) to −175 mV (no. 13) in 15-mV increments. Passive currents have been subtracted. (*b*) Semilogarithmic plot of PSC decay. (Only the linear portions of the decay are used to determine the decay rate constant.) Same time axis applies to (*a*) and (*b*). (*c*) Peak PSC vs. membrane potential. Dashed line intersects V axis at the extrapolated reversal potential (+14 mV) and shows a conductance of 60 mmho/cm². Resting conductance of this cell was 91 mmho/cm², about 50% higher than the average value. Temperature, 15°C.

One likely factor is that at positive potentials the channel duration becomes so short that many channels can close during the growth phase of the PSC. For preparations with intact esterase at 15°C the growth phase lasted about 1.2 ms; the "growth time" (Gage and McBurney, 1975), during which the PSC increased from 20% to 80% of its maximum size, was 0.7 ms. As at amphibian endplates, the growth phase seems to be shaped primarily by temporal dispersion in quantal release, since individual miniature PSCs reach their peaks about four times faster (Lester et al., 1976). Regardless of what causes the prolonged growth phase, it is comparable in length to the average channel lifetime at 0 mV, 0.8 ms ($1/\alpha_0$). If at this voltage a certain number of channel openings occur at a uniform

rate during a PSC growth phase which lasts 1.2 ms, only half the channels remain open by the time the PSC reaches its peak.

If the esterase is blocked, however, ACh molecules remain in the cleft longer and so might reactivate channels which have just closed. In agreement with the arguments presented here, we found that MSF- and prostigmine-treated cells showed more consistent reversals than did cells with intact esterase. Fig. 2 provides a typical example.

There seems to be at least one other cause for the small outward PSCs. We have measured PSCs during the reversible shift in zero-current potentials described by Lester et al. (1975). The shift is induced by bath-applied agonist, by batrachotoxin, and by other treatments that cause Na^+ to replace internal K^+

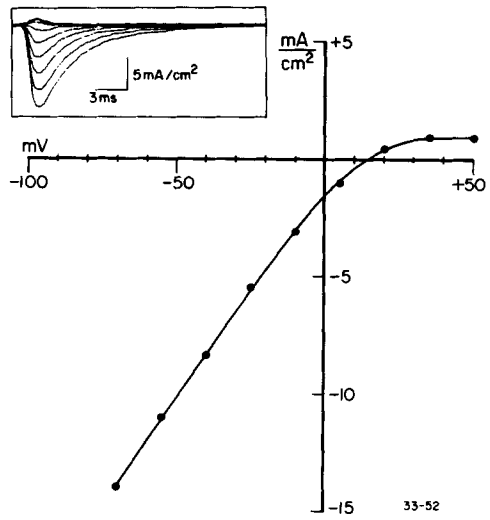

FIGURE 2. Reversal of the PSC in a preparation treated with MSF to inhibit acetylcholinesterase. Reversal occurred at +14 mV. *Inset* shows PSC currents. Temperature, 15°C.

(Lester, 1977); there is presumably also an increased internal Ca^{++}. The results will be described in detail elsewhere but they may be summarized by the following statements. (*a*) During the shift the reversal potential of the PSC remains unaffected, and there are no changes in the kinetics or amplitude of inward PSCs; (*b*) however, there is a marked reversible diminution of outward PSCs.

It is not clear how the ionic redistributions which cause the shift in zero-current potential diminish the size of outward PSCs. Nonetheless, the effect on the acetylcholine receptors at positive potentials can be summarized by the following possibilities. (*a*) The population of channels could have a decreased activation probability, or opening rate; this mechanism is eliminated by the absence of outward tail currents during bath-applied agonist (see below). (*b*) The population of channels might have a greater probability of inactivation—closing rate α—than expected from the trend at negative potentials. (*c*) Individual

channels whose gates are open might have a decreased conductance γ—i.e. rectify instantaneously—for outward currents.

Frog endplate currents display a nonlinear peak I-V plot over a wide voltage range; this has been ascribed to a slightly voltage-dependent channel opening rate (Magleby and Stevens, 1972*b*; Dionne and Stevens, 1975). By contrast, electroplaque PSCs show a linear peak I-V plot at voltages more negative than −30 mV; at more positive voltages the nonlinearity is opposite in direction, and much larger than that of frog endplate currents. In our PSC measurements a slightly voltage-sensitive opening rate would escape detection because of the uncertainties noted here. Nonetheless, the voltage-jump data (see below) suggest that the opening rate varies little, if any, with voltage.

Voltage-Jump Experiments with Bath-Applied Agonist

CONDUCTANCES INDUCED BY BATH APPLICATION OF AGONIST In experiments with bath-applied agonist, we sought to measure steady-state conductances and voltage-jump relaxations over a wide range of conditions. Of particular interest is ACh in the range 300–1,000 μM, since such concentrations are estimated to occur for a fraction of 1 ms during the miniature PSC (Kuffler and Yoshikami, 1975; Fertuck and Salpeter, 1976; Lester et al., 1976) and are thought by some investigators to activate a sizable fraction of the nearby receptors in this short time (Hartzell et al., 1975; Matthews-Bellinger and Salpeter, 1976; Lester et al., 1976; but see Magleby and Stevens, 1972*b*). Unfortunately, when ACh at such high concentrations is flushed through pool A, desensitization occurs while agonist is still equilibrating near the receptors (Lester et al., 1975).

However, the dose-response curve shifts to the left at more negative membrane potentials. As a result even with the agonist concentrations testable by bath application, the agonist-induced conductances approach an upper limit during brief (<1 s) excursions to high negative potentials (Lester et al., 1975). This upper limit resembles the conductance during the PSC: at −175 mV, the largest currents induced by bath-applied ACh were, on the average, 0.77 times those of the neurally evoked PSC (three cells; for a more detailed comparison see Lester et al., 1976). It should be remembered that ACh release was facilitated by frequent stimulation during PSC trials (see Materials and Methods).

We therefore emphasize that although agonist is present for minutes during bath application but for only milliseconds during the PSC, the two experiments give access to roughly the same number of channels. At this point we have no assurance that precisely the same receptor populations are involved—extrasynaptic receptors may figure in the former response, but not in the latter—but it seems justified to compare kinetic data for the two cases.

WITH ONE AGONIST RELAXATIONS ARE EXPONENTIAL Voltage-jump relaxations are measured most precisely with trials like those shown in Fig. 3. In the presence of steady agonist concentration, the voltage is stepped from a positive value—usually +50 mV—to a more negative potential. No steady-state, agonist-induced conductance can be detected at positive potentials (Lester et al., 1975), but the conductance increases gradually during the several milliseconds after the jump. Thus we term such jumps *on*-relaxations. We have analyzed several hundred trials like those of Fig. 3 and in most cases the *on*-relaxation is

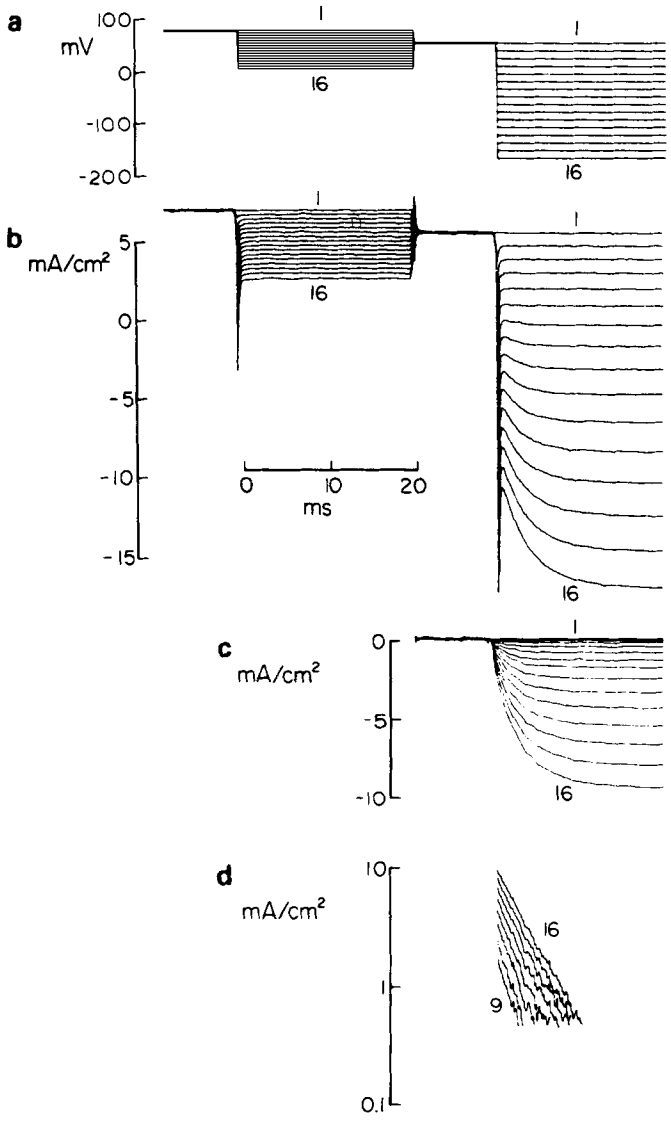

FIGURE 3. Voltage-jump relaxations in 50 μM ACh. Averaged records from two identical trials, each consisting of 16 episodes. (*a*) Membrane potentials during each episode (numbered 1–16). (*b*) Voltage-clamp currents during the same episodes. Currents during the first half of each episode are essentially passive and capacitative (passive conductance was 58 mmho/cm²); currents during the second half contain agonist-induced components. (*c*) Agonist-induced currents obtained by scaling and adding the two halves of each episode (see Materials and Methods). (*d*) Semilogarithmic plot of approach to steady state for the last eight clamp episodes in (*c*). Same time axis applies to all panels. Temperature. 15°C.

described well by a single exponential time course. This statement can be made with most certainty about the jumps with the largest currents and slowest rate constants; for acetylcholine, in the jump from +50 to −175 mV, it is usually

possible to follow the relaxation past 95% of the approach to the final agonist-induced current.

In the PSC experiments there were only small and variable outward currents. Among the causes for this phenomenon we considered the possibility that the conductance γ of an individual channel shows instantaneous inward-going rectification. In *on*-relaxations from a positive to a negative voltage, such rectification might produce an initial rapid increase of agonist-induced current. This fast component would, however, form a small fraction of the relaxation because only a few channels have open gates in the steady state at positive voltages. An obvious fast component was not generally seen in cases where we were confident that passive and gating currents had been accurately subtracted. Nonetheless, the estimated relaxation amplitudes, obtained by extrapolating the single exponential back to the time of the jump, were usually slightly smaller than the actual relaxations. This suggests that a single component did not account for the entire relaxation amplitude. For *on*-relaxations to voltages more negative than −100 mV, the discrepancy rarely exceeded 10%. For voltages more positive than this, the discrepancy sometimes exceeded 20% but the estimate had a similarly large uncertainty. Thus *on*-relaxations yield inconclusive results on the subject of instantaneous rectifications.

In a minority of trials with Sub, we have noted two exponential components, with roughly equal amplitudes and with rates differing by about threefold.

ON-RELAXATIONS HAVE THE SAME TIME CONSTANT AS *OFF*-RELAXATIONS We have presented evidence that the relaxation time constant τ is an instantaneous function of the membrane potential, insensitive to its history or to the history of the conductance (Sheridan and Lester, 1975*a*). The most stringent test of this point is to compare an *on*-relaxation (Fig. 3) with one leading to a conductance decrease (*off*-relaxation), and with the same final voltage for each jump. Records from an *off*-trial are shown in Fig. 4. Such relaxations can be measured with less precision than can *on*-relaxations, since they take place against a background of initially high conductance and since the final potential represents only a moderate electrochemical driving force. In one series of experiments we compared *on*- and *off*-relaxations with the same final voltage. The relaxations could be followed past 90% of the approach to steady state. For all agonists and concentrations tested, the time constants for the two relaxations agreed within the limits of error (Table I).

THE INSTANTANEOUS CURRENT-VOLTAGE PLOT IS LINEAR AT NEGATIVE VOLTAGES We have already described how *off*-relaxations provide information on the I–V characteristics of open ion channels (Lester et al., 1975). In the present experiments the instantaneous I–V plot has been studied for ACh, Sub, and Carb at temperatures from 8°C to 15°C. In all cases the curve was linear at potentials more negative than about −30 mV. The reversal potential for the agonist currents was obtained by extrapolating the instantaneous I–V plots to zero current; the reversal potential ranged from 0 to +20 mV and did not vary with the agonist or its concentration. These observations confirm earlier data (Lester et al., 1975) and agree well with the reversal potentials for neurally evoked postsynaptic currents.

The steady-state agonist-induced currents are too small to measure at positive potentials (Lester et al., 1975). For instantaneously linear channels, one would nonetheless expect *off*-relaxations to show a brief outward "tail" of current when the final voltage is more positive than the reversal potential. We have rarely seen

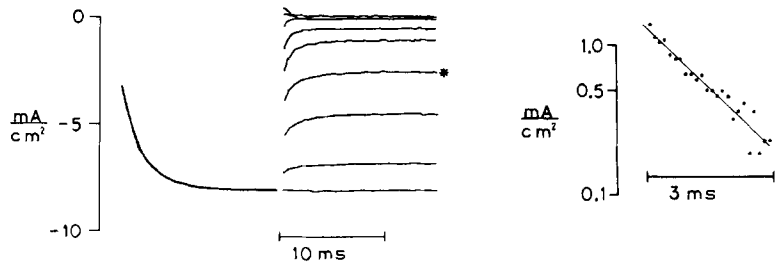

FIGURE 4. *Left*, *Off* voltage-jump relaxations in ACh. 50 μM. Agonist-induced currents are shown. For episode shown in lowest trace, voltage remained at −200 mV. For other episodes, voltage was jumped from −200 mV to more positive values. The second value ranged from −184 mV to +40 mV, in increments of 32 mV. For clarity, traces are blanked out for 360 μs after the jump. *Right*, Semilogarithmic plot of the approach to steady state for the jump from −200 mV to −120 mV (indicated by the asterisk). Plot starts after same delay (360 μs). Temperature, 15°C.

TABLE I

COMPARISON OF *ON* AND *OFF* RELAXATIONS

Cell	Agonist	$1/\tau$ *Off*	$1/\tau$ *On*
	μM	ms^{-1}	ms^{-1}
20-32	ACh, 55	0.25±0.02	0.24±0.02
	Carb, 50	0.42±0.03	0.39±0.04
20-41	ACh, 75	0.43±0.03	0.43±0.01
20-51	ACh, 75	0.38±0.03	0.35±0.03
	Carb, 50	0.61±0.05	0.63±0.05
26-61	Sub, 3	0.12±0.01	0.12±0.01
	Sub, 5	0.20±0.02	0.19±0.01

Reciprocal time constants, $1/\tau$, for voltage-jump relaxations to a final voltage of −100 mV. Starting voltage was −175 mV for *off* jumps, +50 mV for *on* jumps. SE of each observation is given. Temperature, 9°C.

such outward tails; even where they occurred (Fig. 4 is our clearest example), they probably included a large contribution from Na channel gating currents and were too small to be consistent with an ohmic channel. The clamping circuit could probably have resolved such tails if they existed since inward sodium current tails were readily measurable (see Materials and Methods). Therefore we conclude that with bath-applied agonist, at least, the instantaneous I-V relation shows a decreased conductance for outward currents. This result fits with the observation (described briefly above) that the neurally evoked PSC does not

reverse during the shift in zero-current potentials provoked by bath-applied agonists and by certain other treatments. However, as noted above, we cannot tell whether the instantaneous nonlinearity arises because individual channels rectify (γ nonlinear) or because at positive potentials the population of channels

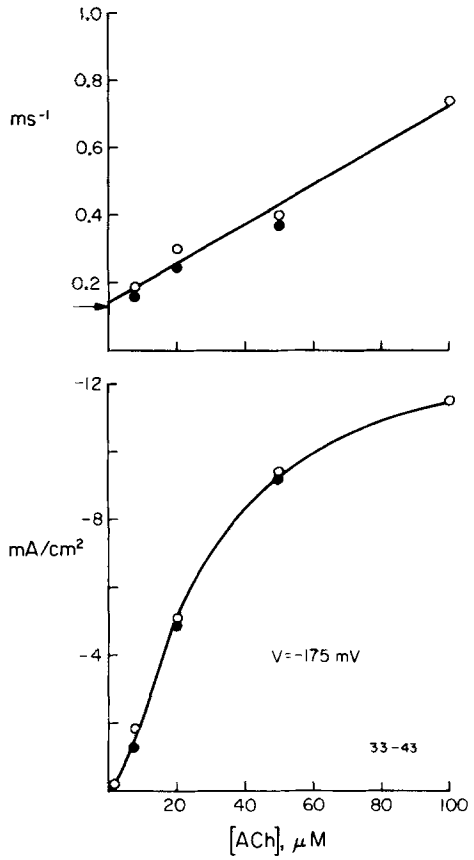

FIGURE 5. Equipotential dose-response relations for one cell. *On* voltage jumps from +50 mV to −175 mV (see Fig. 3). *Top*, Relaxation rate constants $1/\tau$ vs. ACh concentration. The arrow indicates the PSC decay rate. α, at the same voltage. *Bottom*, Steady-state agonist-induced current vs. ACh concentration. Closed circles, first concentration series; open, second series. Temperature, 15°C.

has a much greater probability of inactivation—closing rate α—than expected from the trend at negative potentials.

RECIPROCAL RELAXATION TIMES DEPEND LINEARLY ON AGONIST CONCENTRATION With many cells, reproducible records were obtained with repeated applications of agonist solutions. With such cells we have studied the effect of agonist concentration on relaxation kinetics. Over a wide range of agonist concentrations, the reciprocal time constants $1/\tau$ increase linearly with agonist concentration (Fig. 5). The constant of proportionality does not change with potential (Fig. 6) but does differ among the various agonists. There is a non-zero intercept

at zero agonist concentration; this intercept varies with potential (Fig. 6) and with the nature of the agonist (Sheridan and Lester, 1975*a*). For the case of acetylcholine, we have compared this intercept with the rate constant α for PSC decays (Fig. 5). For a given cell and a given potential, the two values were equal within experimental error (±20%). Nonetheless, on the average α exceeded the zero concentration intercept by about 15%. We are not sure whether this finding points to a real difference between the two kinds of kinetic data.

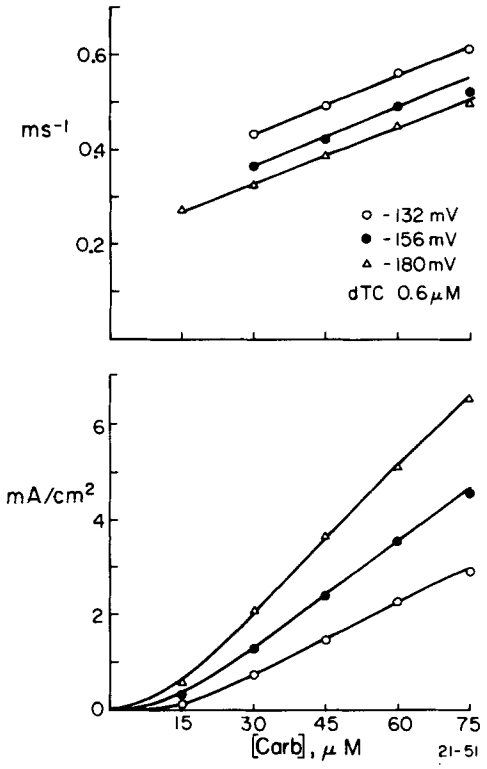

FIGURE 6. Equipotential dose-response relations in the low-conductance region. *Top*, Relaxation rate constant $1/\tau$ vs. Carb concentration; *bottom*, steady-state agonist-induced current vs. Carb concentration. *On*-relaxations (see Fig. 3). Final voltages: ○, −132 mV; ●, −156 mV; △, −180 mV. Data were averaged from an ascending and a descending concentration series. Data were taken in the presence of *d*TC, 0.6 μM. Temperature, 15°C.

PRELIMINARY INTERPRETATION OF KINETIC DATA There is apparently little or no ACh remaining near the receptors during the falling phase of the PSC, because few channels open during this phase (Magleby and Stevens, 1972*a*,*b*; Anderson and Stevens, 1973; Gage and McBurney, 1975). This reasoning underlies the interpretation of the PSC decay rate constant as the rate constant α for channel closing (see Introduction). A voltage-jump relaxation is however performed with finite agonist concentration and channels can therefore both open and close during the relaxation. In such a case one expects that the reciprocal time constants $1/\tau$ (Figs. 5 and 6) equal the sum of two components, the channel

closing rate α plus the channel opening rate. Since the opening rate presumably decreases to zero at zero agonist concentration, it is hardly surprising that, for acetylcholine, the zero concentration intercept of $1/\tau$ nearly equals the PSC decay rate α (Fig. 5). Furthermore, the simplest possible hypothesis is that the closing rate does not vary with the agonist concentration. On this assumption, the opening rate is that component of $1/\tau$ which increases with agonist concentration.

The scheme just given allows one to reconstruct the behavior of the channel population during sustained or brief exposure to agonist (Sheridan and Lester, 1975*a*; Lester et al., 1976; see Discussion). For the moment we point out merely that this interpretation, if correct, demands that conditions resulting in equal opening and closing rates should also produce half the maximal steady-state conductance. In the cell of Fig. 5, the opening and closing rates are both roughly equal to 0.15 ms^{-1} at 25 μM ACh (Fig. 5), and roughly half the saturating current seems to be measured at this concentration (Fig. 5). Most cells tested at the temperature (15°C) and voltage (−175 mV) of Fig. 5 were more responsive to ACh: the opening rate increased to 0.15 ms^{-1}, and half-maximal conductances were observed, at 10–15 μM ACh.

Conductances level off at high concentrations of agonist but the opening rate does not (Fig. 5). It may immediately be inferred that saturation of the opening rate does not limit the conductance. Another important conclusion is that the opening rate does not depend strongly on voltage, since in Fig. 6 the concentration-dependent part of $1/\tau$ does not vary with voltage. As expected from the parallel lines in Fig. 6 the voltage sensitivity of $1/\tau$ is entirely accounted for by that of α: when α is subtracted from $1/\tau$, the resulting value varies little if any with voltage (Sheridan and Lester, 1975*a*).

LOW AGONIST CONCENTRATIONS In order to decide among various theories to explain the nonlinear increase of conductance with agonist concentration, we sought to measure how the kinetics behave for very small responses, in the initial nonlinear region of the dose-conductance curve. This region was explored either with low agonist concentration or, as in Fig. 6, the curve was shifted to higher agonist concentrations with the competitive antagonist *d*-tubocurarine (*d*TC). Despite the use of averaging techniques, the currents were too small to allow measurement of relaxation time constants.

EFFECT OF ANTAGONISTS Agonist-induced conductances are reduced by several classes of drugs including curare, the irreversible snake venom toxins, and local anesthetics (Lester et al., 1975). Prolonged exposure to agonists themselves produces desensitization. We have studied how some of these treatments affect the relaxation rate constants. Bungarotoxin has a simple effect on carbachol-induced conductances: the steady-state conductances are reduced with no change in the relaxation time constants (Fig. 7).

Curare has a different effect. Relaxations still follow a single exponential time course, but the reciprocal time constants decrease as well as the steady-state conductances. There is little or no effect on the voltage-dependence of the time constants (Fig. 8). The effect of *d*TC on equilibrium conductances can be overcome by increasing the agonist concentration, and the same is true for curare's effect on the time constants. For instance, in the presence of 0.2 μM

*d*TC, both equilibrium conductances and time constants were restored approximately to their control values by doubling the agonist concentration (Fig. 8).

Further experimentation has failed to confirm our earlier report that the relaxation kinetics seem to change during desensitization (Sheridan and Lester, 1975*a*). The observed effects probably arose from temperature changes in the chamber during solution flow, rather than from densensitization. At this time we cannot provide an accurate description of the relaxation kinetics during desensitization.

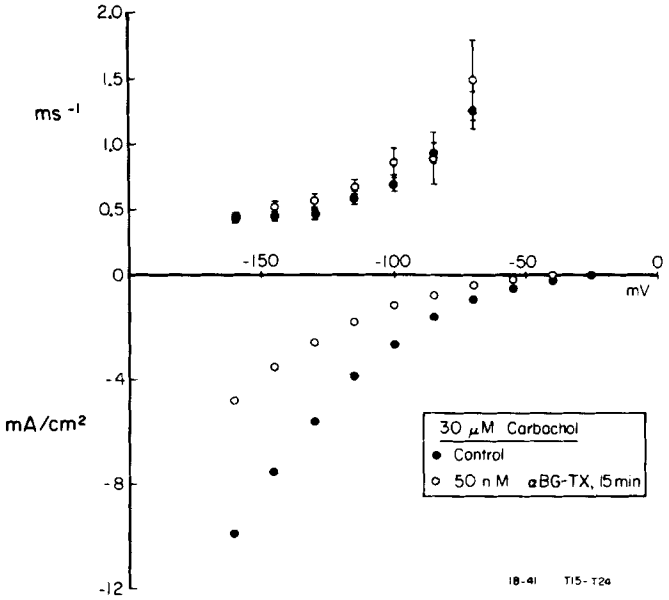

FIGURE 7. Effects of α-bungarotoxin on responses to Carb. 30 μM. *On*-relaxations. Equimolar plots of (*top*) relaxation rate constant. $1/\tau$, and (*bottom*) steady-state agonist-induced current vs. membrane potential. Symbols: ●, control responses; ○, after treatment with 50 nM α-bungarotoxin for 15 min. Error bars give SE of rate measurements. Temperature, 15°C.

IN THE PRESENCE OF TWO AGONISTS, RELAXATIONS HAVE A COMPLEX TIME COURSE In the experiments discussed thus far, we observed no major departures from a simple exponential time course. Such departures do occur, however, under several conditions, including simultaneous exposure to two different agonists. Complex decays have been observed for the following solutions: ACh (10 μM) + Deca (6 μM); ACh (5 μM) + Carb (50 μM); Sub (5 μM) + Carb (100 μM) (Fig. 9). In each case, relaxations need to be described by at least two exponential components, but the matter calls for further investigation.

We have observed rather complex nonexponential relaxations with the bromide and methylsulfate salts of prostigmine, with eserine, and with certain local anesthetics (D. D. Koblin, R. E. Sheridan, and H. A. Lester, unpublished results). P. R. Adams (personal communication) has made similar observations at frog endplates.

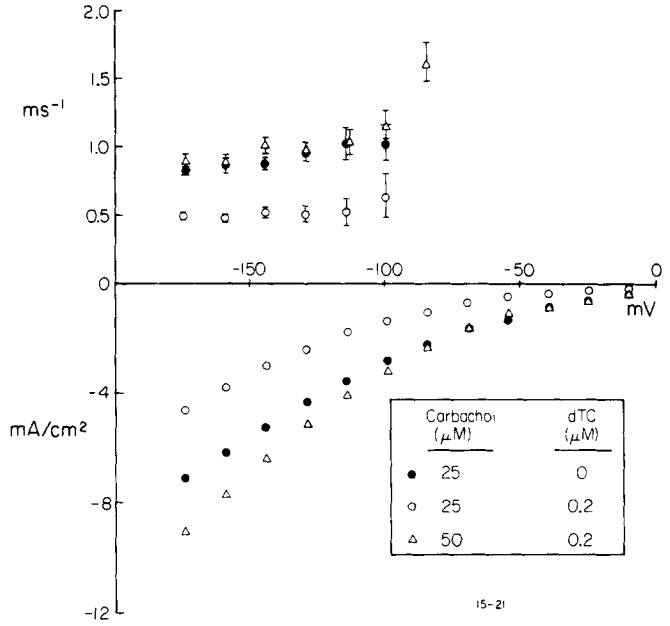

FIGURE 8. Competitive interaction of *d*TC and agonist. *Top*, Relaxation rate constant, $1/\tau$, and (*bottom*) steady-state current induced by Carb vs. membrane potential. ●, control responses to 25 μM carbachol alone. ○, responses to 25 μM carbachol after equilibration in 0.2 μM *d*TC. △, responses to 50 μM carbachol after equilibration in 0.2 μM *d*TC. Error bars give SE of rate measurements. Temperature, 15°C.

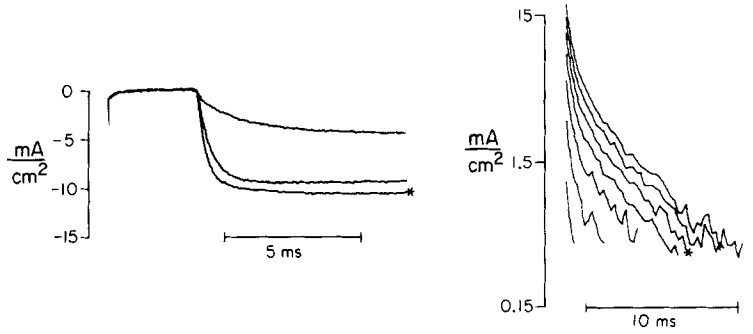

FIGURE 9. Voltage-jump relaxations in 5 μM Sub and 100 μM Carb. Asterisk shows the same episode in both panels. *Left*, *On*-relaxations from +50 mV to −135 mV (see Fig. 3*c*). Upper trace, Sub alone. Middle trace, Carb alone. Lower trace, both agonists applied simultaneously. *Right*, Semilogarithmic plot, starting 200 μs after the jump, of the approach to steady state in the presence of both agonists. Final membrane potential ranges from −15 to −195 mV in 30-mV increments (see Fig. 3*d*). Temperature, 15°C.

Voltage Sensitivity of the Steady-State Conductance

Whatever the molecular nature of the events which open and close channels, the data suggest that at negative potentials the steady-state conductance depends on

voltage simply because the channel closing rate α depends on voltage, with little or no added voltage-sensitivity of the opening rate constant. When the opening rate is much less than the closing rate, the agonist-induced conductance is small and depends exponentially on membrane potential (Lester et al., 1975). The PSC decay rate also depends exponentially on voltage. If the hypothesis is correct, the voltage dependencies should be equal (Dionne and Stevens, 1975). In two experiments to test this point the esterase was left intact. First, PSCs were measured. Next, acetylcholine (1–5 mM) was added to the bath and voltage jumps were measured as usual. The ACh was then washed out and PSCs were again measured. In four other experiments the PSCs were measured and the esterase was then blocked with MSF. Finally, voltage jumps were measured with bath-applied ACh at low concentrations. We used data for voltages more nega-

TABLE II

VOLTAGE DEPENDENCE OF PSC DECAY RATE (α) AND OF SMALL STEADY-STATE CHORD CONDUCTANCE (G_c) INDUCED BY ACh

	mV for e-fold change	
Cell	α	G_c
31-23*	66	82
31-43*	112	104
31-52	81	86
33-13	74	88
33-43	94	89
33-52	94	75
Mean ± SD	87 ± 16	87 ± 10

* Experiments where high concentrations of ACh were used to overcome acetylcholinesterase. For others, the esterase was inhibited with MSF. Both α and G_c depended exponentially on membrane potential (Lester et al., 1975; Sheridan and Lester, 1975 *a*). Temperature, 15°C.

tive than −30 mV in order to remain in the region of linear channel conductance. The results of both types of experiments are summarized in Table II. Within the experimental limits of roughly 10%, small steady-state chord conductance has the same voltage dependence as does α.

DISCUSSION

The present data concern the kinetics and amplitudes of agonist-induced conductances and the effects of agonist concentration, membrane voltage, and pharmacological agents. The data seem to bear on rate-limiting steps at the acetylcholine receptor itself. Many possible extraneous causes for the time-dependent conductances, e.g., time-varying concentrations of ions or agonist near the membrane, seem unlikely because the rate constants are insensitive to the history of the system (however, the case of desensitization remains to be explored) and to partial inactivation of the response by α-bungarotoxin.

At this point it appears worthwhile to ask whether these data suggest a mechanism of response to nicotinic agonists. We cannot, of course, decide unambiguously on such a mechanism because we do not know the time course of agonist binding to the receptor, which constitutes the first step in the process.

This point has been extensively discussed by Magleby and Stevens (1972*a*, *b*), by Kordas (1972*a*, *b*), and by Gage and McBurney (1975). Nonetheless, at negative potentials the agonist-induced chord conductance provides a linear measure of the open ion channels which finally result from this binding.

We begin by briefly pointing out the ways in which the data constrain any models. (*a*) The relaxation time constants themselves have a simple description in terms of classical relaxation theory (cf. Eigen and DeMaeyer, 1974). Channels seem to open at a rate that is linear with agonist concentration, implying that a bimolecular reaction between agonist and receptor either precedes or constitutes the rate-limiting step in channel opening. (*b*) The lifetime of the channel varies with membrane potential, implying that whatever event closes the channel, its rate of occurrence is voltage dependent (Magleby and Stevens, 1972*a*, *b*; Kordas, 1972*a*, *b*; Gage and McBurney, 1975). (*c*) Small steady-state conductances have approximately the same voltage dependence as the channel closing rate α, in agreement with the finding that the opening rate has little or no additional voltage sensitivity (Dionne and Stevens, 1975; Sheridan and Lester, 1975*a*). (*d*) With one agonist, relaxations usually followed an exponential time course, even when they involved large departures from equilibrium. For instance, some of the *on*-relaxations represented a change from <1% to >50% of the maximal conductance. Although many models give exponential relaxations for infinitesimal perturbations, a successful model in this case must predict exponential relaxations for large perturbations as well. (*e*) Finally, despite the simple linear concentration dependence of the reciprocal time constants $1/\tau$, the equilibrium conductance depends nonlinearly on the agonist concentration.

MODELS

One Agonist, One Receptor: Inadequate but Instructive Models

CHANNEL OPENED DIRECTLY BY BINDING As noted above, we are ignorant of possible transient or steady-state conditions when a receptor molecule has agonist bound but when its associated channel remains closed. The simplest possible molecular model would be one in which such intermediates do not occur, i.e., the channel opens when an agonist binds and closes again when it dissociates.

$$A + R \underset{k_{-b}}{\overset{k_{+b}}{\rightleftharpoons}} AR^*. \tag{1}$$

Here A is the agonist molecule, R is the receptor, and AR^* is the bound state with open channel. This simple model predicts exponential PSCs; voltage-jump relaxations are also exponential if, *as we assume throughout this Discussion, the agonist concentration near the receptors is instantaneously buffered by the bulk solution.* The relaxation time constant τ is given by

$$1/\tau = k_{+b}[A] + k_{-b}. \tag{2}$$

The channel closing rate, the agonist dissociation rate, and the PSC decay rate are all given by $\alpha = k_{-b}$. The right side of Eq. (2) is the sum of this presumably voltage-sensitive closing rate and an opening rate which depends linearly on

agonist concentration. This formulation fits the time constant data. However the model demands a strictly hyperbolic dose-conductance relation:

$$I = r\gamma[V - V_0]\left(\frac{k_{+b}[A]}{k_{+b}[A] + k_{-b}}\right), \tag{3}$$

where r is the total number of receptors, γ the conductance of an open ion channel, and V_0 the reversal potential. Since the dose-conductance relation has a sigmoid start, we must reject this model.

RATE-LIMITING BINDING In two other, possibly more realistic versions of this model, the channel is opened and closed by an intramolecular isomerization which follows the binding step. Neither version predicts a sigmoid dose-conductance relation, but we treat both briefly to show how the nature of the rate-limiting step affects the kinetics.

$$A + R \underset{k_{-b}}{\overset{k_{+b}}{\rightleftharpoons}} AR \underset{k_{-c}}{\overset{k_{+c}}{\rightleftharpoons}} AR^*. \tag{4}$$

Here AR is an intermediate, inactive state. Whatever the rate-limiting step, the dose-conductance curve becomes:

$$I = r\gamma[V - V_0]\left[\frac{k_{+c}k_{+b}[A]}{k_{+b}[A]k_{+c} + k_{+b}[A]k_{-c} + k_{-b}k_{-c}}\right]. \tag{5}$$

Exponential relaxations and PSCs occur if steps b (binding) and c (conformational change or channel opening) differ greatly in speed. For instance, suppose that binding of the agonist to the receptor is much slower than the subsequent isomerization ($k_{\pm c} \gg k_{\pm b}$). Then the relaxation rate constants fit the observed data:

$$1/\tau = k_{+b}[A] + k_{-b}\left(\frac{k_{-c}}{k_{-c} + k_{+c}}\right). \tag{6}$$

The closing rate α is the second term on the right; its voltage dependence might reside either in the dissociation rate k_{-b} or in the rapid isomerization. In the latter case the ratio $k_{+c}/(k_{+c} + k_{-c})$ must be near unity under conditions where linear instantaneous I-V relations are measured: at all voltages for frog endplates (Magleby and Stevens, 1972*b*; Dionne and Stevens, 1975) and at least at negative voltages for electroplaques (this point was brought to our attention by P. R. Adams). Such a situation is equivalent to Eq. (1).

With the type of experiment described in this paper we cannot distinguish between Eq. (2) and (6) or between Eq. (3) and (5). However, partial agonists might have a low isomerization ratio $k_{+c}/(k_{+c} + k_{-c})$. This suggestion could be checked with measurements on single-channel currents (Neher and Sakmann, 1976*a*), since the apparent channel conductance in such experiments would be γ times this ratio.

RATE-LIMITING ISOMERIZATION If, in the model of Eq. (4), the isomerization or conformational change is itself the rate-limiting step ($k_{\pm b} \gg k_{\pm c}$), the predicted relaxation rates are

$$1/\tau = k_{+c}\frac{k_{+b}[A]}{k_{+b}[A] + k_{-b}} + k_{-c}, \tag{7}$$

with $k_{-c} = \alpha$ as the voltage-sensitive closing rate. There is an important distinction between this model and one in which binding is rate-limiting (Eq. [2] and [6]). Eq. (7) demands that reciprocal relaxation times $1/\tau$ level off at high agonist concentrations. A final choice between the two models, based on this test, must await techniques to abolish desensitization. Nonetheless, in our data there is no indication that $1/\tau$ levels off for agonist concentrations too low, or for times too short to desensitize many receptors. Thus although we cannot definitely decide whether binding or isomerization is rate limiting, in either case $k_{+c} + k_{-c}$ must exceed the fastest observed $1/\tau$ (roughly 2 ms^{-1} at 15°C and 4 ms^{-1} at 22°C). Furthermore, in the case of rate-limiting conformation change, the fast binding step must have a dissociation constant k_{-b}/k_{+b} in excess of 100 μM for ACh, 250 μM for Carb, or 2 μM for Sub. It will also be noted that in Eq. (5) the "apparent" dissociation constant, or agonist concentration for half-maximal response, is $(k_{-b}/k_{+b})k_{-c}/(k_{-c} + k_{+c})$. In our experiments at −150 mV the apparent K_D for ACh was 10–15 μM; we conclude that if isomerization is rate limiting under these conditions, $k_{-c}/(k_{-c} + k_{+c})$ does not exceed 0.15. These considerations about relaxation kinetics apply to any model, such as the concerted and sequential mechanisms discussed below, in which the rate-limiting step is either binding of agonist or an intramolecular isomerization.

Dimerization Is Not the Rate-Limiting Step

A voltage-dependent dimerization of gramicidin molecules apparently determines the conductance of artificial membranes containing this antibiotic (Bamberg and Laüger, 1973; Zingsheim and Neher, 1974). If this mechanism governed the acetylcholine receptor, the agonist-receptor binding would proceed as in the previous models:

$$A + R \underset{k_{-b}}{\overset{k_{+b}}{\rightleftharpoons}} AR.$$

Dimerization of AR molecules would follow:

$$2\,AR \underset{k_{-d}}{\overset{k_{+d}}{\rightleftharpoons}} (AR)_2.$$

The $(AR)_2$ state would comprise, or rapidly equilibrate with, the open ion channel. At equilibrium the following condition is satisfied:

$$k_{+d}\left[\left(\frac{k_{+b}[A]}{k_{+b}[A] + k_{-b}}\right)(r - 2\,[(AR)_2])\right]^2 = k_{-d}[(AR)_2]. \tag{8}$$

From this it may be concluded that the dose-conductance curve is sigmoid, with a half-maximal point at

$$[A] = \frac{k_{-b}/k_{+b}}{(r - p)(2k_{+d}/pk_{-d})^{\frac{1}{2}} - 1}, \tag{9}$$

where

$$p = r/2 + k_{-d}/8k_{+d} - [8rk_{-d}/k_{+d} + (k_{-d}/k_{+d})^2]^{\frac{1}{2}}/8. \tag{10}$$

However, whichever step is rate limiting, the kinetic data do not fit the predictions. For instance, suppose that $k_{\pm b} \gg k_{\pm d}$; i.e., the binding step is at

equilibrium and the dimerization is rate limiting. For this scheme the relaxations are indistinguishable from simple exponentials as long as rk_{+d}/k_{-d} is less than about 3, implying that at the highest concentrations of agonist only about $^1/_3$ of the receptors are in the dimer form associated with the open channel (Bamberg and Laüger, 1973). Furthermore, reciprocal relaxation times $1/\tau$ increase with [agonist]. However, in disagreement with the data, as conductance levels off, $1/\tau$ does so also. The concentration-dependent term is roughly hyperbolic, with a half-maximal concentration close to that given by Eq. (9).

If the binding is the rate-limiting step ($k_{\mp d} \gg k_{\mp b}$), *on*-relaxations differ markedly from a simple exponential time course.

Concerted Transitions Do Not Fit the Data

This model, first proposed by Monod et al. (1965) and applied to the ACh receptor by Karlin (1967*a*), postulates that the receptor is an oligomeric protein and that all its component protomers simultaneously undergo the transitions between the inactive (R_T), or "channel closed" and active (R_R) or "channel open" states. Each protomer in the R_R state displays the same microscopic rate and equilibrium constants for agonist binding; a lower affinity characterizes the R_T state. This model's relaxation properties have been analyzed in some detail (Kirschner et al., 1966; Colquhoun and Hawkes, 1977; P. R. Adams, personal communication), but since the model fails to account for the data, we merely summarize the conclusions here.

If the rate-limiting step consists of binding of the agonist to either the R_R or the R_T state, then relaxations would be measurably nonexponential. If, on the other hand, the $R_R \rightleftharpoons R_T$ isomerizations are rate limiting, then exponential relaxations occur. Furthermore, $1/\tau$ increases with agonist concentration if the $A_nR_T \rightarrow A_nR_R$ isomerization has a higher rate constant as n increases. Such a situation seems physically plausible even though it differs (*a*) from the findings of Kirschner et al. (1966) on glyceraldehyde-3-phosphate dehydrogenase, and (*b*) from the assumptions of the theoretical treatment by Hammes and Wu (1974). However, the model demands that both the equilibrium conductance and the reciprocal relaxation times depend in the same fashion on agonist concentration; i.e., that the equipotential dose vs. conductance curve and dose vs. $1/\tau$ curve in Fig. 5 have a similar, sigmoid shape. The data disagree with this prediction as well as with the demand that for a given voltage the channel duration and the zero-concentration relaxation times have a common value for all agonists (Sheridan and Lester, 1975*a;* Neher and Sakmann, 1976*a*).

Sequential Models Account for the Data

These schemes are based on another general theory for cooperative activity in proteins (Koshland et al., 1966). Channel activation results from the interaction of two agonist molecules with the receptors; *the second interaction constitutes the rate-limiting step*. Each interaction presumably consists of a binding (*b* step) followed by a conformational change (*c* step) as in scheme (4), but we write each interaction as though it follows the simpler scheme (1) in which the binding and conformational change occur simultaneously. In this scheme the forward reaction flux (number of interactions/second) increases linearly with [agonist]. Thus

we write the model as though the b step limits the rate of each interaction; but a slow c step, preceded by a rapid b step, can be ruled out only to the extent that we have given upper limits on the affinity of the rapid binding (see discussion of scheme 4, above).

We assume that the receptor protein has two agonist binding sites and that the first and second bindings are characterized by roughly the same equilibrium affinity.

$$2A + R \underset{k_{-1}}{\overset{2k_{+1}}{\rightleftharpoons}} A + AR \underset{2k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} A_2R. \tag{11}$$

In scheme (11) the statistical factors of 2 arise because the sites are equivalent when both or neither are occupied by agonist. Within the framework of this scheme, the observation of simple exponential relaxations implies that R and AR are always in equilibrium on the time scale of our experiments. The temporal resolution of our measurements allows the conclusion that k_{+1}/k_{+2} and k_{-1}/k_{-2} both exceed about 5. In one of many possible physical pictures of this scheme, the second binding and dissociation might proceed more slowly than the first because binding to either site results in a small conformational change that hinders the passage of agonist molecules between the solution and the other site.

For the agonists and conditions studied here, the A_2R state seems to contribute substantially all the conductance; otherwise the relaxations would have rapid components and the dose-conductance curve would have a linear start. Thus the dissociation rate constant $2k_{-2}$ is assumed to depend on voltage and to equal the experimentally measured closing rate α;

$$2k_{-2} = \alpha = \alpha_0 e^{V/V_1}. \tag{12}$$

The relaxation times τ are given by:

$$1/\tau = \frac{k_{+2}[A]^2}{[A] + k_{-1}/2k_{+1}} + 2k_{-2}; \tag{13}$$

and the equilibrium currents by:

$$I = r\gamma(V - V_0)\left[\frac{[A]^2}{[A]^2 + 2[A]k_{-2}/k_{+2} + (k_{-1}k_{-2})/(k_{+1}k_{+2})}\right]. \tag{14}$$

Of the models described above and of several more we have explored, only the sequential model accounts simultaneously for the observed Hill coefficients of nearly unity for the relation between dose and reciprocal relaxation time and of nearly two for that between dose and steady-state conductance. The case of ACh provides the model's most rigorous test, since definite predictions are made about PSC rates, voltage-jump relaxations, and equilibrium conductances. The total maximum conductance $r\gamma$ was estimated by the slope of the I-V plots (Fig. 10) at high agonist concentrations and high negative potential; $r\gamma$ ranged from 70 to 200 mmho/cm^2. Thus there are no free parameters. However, as already pointed out (Lester et al., 1975), the values for $r\gamma$ are much smaller than would be expected from the measured density of elapid α-toxin binding sites in the membrane.

The model accounts well for the data at negative potentials. Although any particular example (e.g., Fig. 10) shows some scatter about the theoretical lines,

there is no systematic deviation among the five cells for which we have similarly complete data. However, at positive potentials the model consistently predicts larger outward currents than are observed. This discrepancy is probably connected with the observed nonlinearities in the instantaneous I-V relation. As

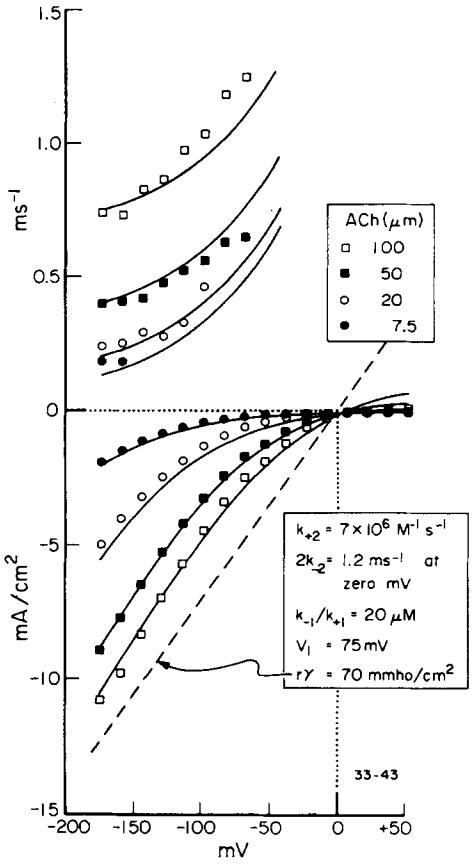

FIGURE 10. Comparison of theory and data for the sequential model. Experiment of Figs. 3, 4, and 5. *Top*, Voltage-jump relaxations. Predicted curves (solid lines) from Eq. (13) with the specified parameters. *Bottom*, Steady-state current induced by agonist. Solid lines are based on Eq. (14) with the same parameters as above; the assumed reversal potential. V_0, is 0 mV. The maximum conductance, $r\gamma$, is taken to be 70 mmho/cm² as shown by the dashed line. Concentrations of ACh are as indicated.

noted in Results, such nonlinearities might indicate: (*a*) that at positive voltages α increases more rapidly than indicated by Eq. (12); or (*b*) that γ decreases for outward currents.

IDIOSYNCRACIES OF THE SEQUENTIAL MODEL According to Eq. (13), the relation between concentration and $1/\tau$ has a nonlinear start at low agonist concentrations. In our experiments, this effect would escape notice (cf. Fig. 6). However, by the fluctuation-dissipation theorem, τ should equal the time constant for decay of the autocorrelation function in fluctuation measurements

(Stevens, 1972). Fluctuation analysis can readily be performed with low agonist concentrations. With such measurements P. R. Adams and B. Sakmann (personal communication) have recently confirmed the prediction that the curve of reciprocal autocorrelation time as a function of [ACh] is concave upward at low ACh concentrations. There is a linear increase at higher concentrations, in agreement with our data on reciprocal relaxation times.

The predicted concentration vs. $1/\tau$ relation is approximately linear at moderate and high concentrations such as those of our studies. However, the predicted *empirical* zero-concentration intercept α_e is some 20–40% smaller than the actual molecular transition rate $2k_{-2}$; the latter is directly measured as the PSC decay rate. Such a discrepancy was noted in most of our experiments but could also have arisen from systematic errors. In any case, the predicted equilibrium

TABLE III

VALUES OF k_{+2} AND $2k_{-2}$ FOR THE SEQUENTIAL MODEL, Eq. (12–14)

Agonist	k_{+2}	$2k_{-2}$ (ms^{-1})	
		0	−175
	$M^{-1}s^{-1}$	*mV*	*mV*
Deca	2×10^8	3.2	0.50
Carb	10^7	1.8	0.25
ACh	10^7	1.0	0.16
Sub	4×10^7	0.6	0.1

The forward rate constant, k_{+2}, is the slope of the equipotential dose-response curve relating [agonist] and reciprocal relaxation time $1/\tau$ (Fig. 5). The reverse rate constant, $2k_{-2}$, is the zero-concentration intercept of the same plot. All values are for 15°C. Standard errors are ±30% for k_{+2}; ±20% for $2k_{-2}$.

conductance may be expressed as a function of α_e and of the reciprocal relaxation time $1/\tau$. This expression in turn is well approximated by a power function of α_e and $1/\tau$, which provided an empirical description of the data in an earlier paper (Sheridan and Lester, 1975*a*). In that paper, α_e and $1/\tau$ were called α and k.

SURVEY OF AGONISTS The sequential model also describes the relaxation time constants and equilibrium currents for the other agonists we have explored (Table III). In fitting the other agonists, we have assumed that the channel closing rate, $2k_{-2}$, has identical voltage sensitivity for all agonists at a given temperature (*e*-fold for 86 mV at 15°C). This constraint arises from the observation that small conductances depend exponentially on V and that the dependence is the same for all agonists (Rang, 1973; Lester et al., 1975; Sheridan and Lester, unpublished observations).

The rate constant for the first binding, k_{+1}, is postulated to exceed k_{+2} by at least fivefold. With the exception of Deca (perhaps a special case; see next paragraph), the postulated values for k_{+1}, k_{+2} thus range from 10^7 to at least 2×10^8 M^{-1} s^{-1}, which typifies the interaction of small ligands with specific binding sites on proteins. For instance, ACh itself binds to acetylcholinesterase with a rate constant of 1.6×10^8 M^{-1} s^{-1} at 25°C (Rosenberry, 1975). Therefore the data seem compatible with the idea that the binding and dissociation steps themselves

limit the rate of channel activation, as suggested by Kordas (1972*a*, *b*). Indeed, if one accepts our interpretation that the reciprocal relaxation time $1/\tau$ consists of a concentration-independent closing rate plus a concentration-dependent opening rate, then our discussion of Eq. (7) is quite generally applicable. Specifically, if any model postulates that the rate-limiting step is an isomerization or conformational change, the model must also postulate that all agonist-receptor bindings occur faster than the values for k_{+2} in Table III.

It will be noted in Table II that the two *bis*-quarternary ammonium compounds, Sub and Deca, have substantially higher forward binding rate constants k_{+2} than do the monoquarternary compounds Carb and ACh. In other experiments we have noted that this pattern extends to several other agonists, including a *bis*-quarternary azobenzene derivative (Lester and Chang, 1977) and the singly charged form of nicotine. It seems reasonable to suggest that the binding reaction is accelerated by coulombic forces. However, in connection with the very high k_{+2} value for Deca it should also be noted that the dose-response curve begins with a Hill coefficient of 1.5 rather than the value of nearly 2 found for Carb and ACh (Lester et al., 1975; and unpublished results). These observations raise the possibility that the presence of just one bound Deca is sufficient to open the channel, so that k_{+2} would in fact represent the rate constant for the first faster binding. Another possibility is that Deca acts partially like a local anesthetic. The matter deserves further study.

In contrast to the forward binding rate constant k_{+2}, the dissociation rate $2k_{-2}$ does not vary systematically with the charge on the agonist but does depend on the membrane potential. Consequently, there is a voltage-dependent equilibrium binding constant for agonists; on the other hand the competitive antagonist *d*TC has the same equilibrium dissociation constant at all voltages even though *d*TC seems to bind to the same site as agonists (Lester et al., 1975). Many investigators have suggested that the agonist-receptor complex, but not the antagonist-receptor complex, is associated with a conformational state of the receptor protein which constitutes or stabilizes the open state of the channel. It is also suggested that the energy barrier for channel closing is modified by the interaction of a dipole moment with the membrane field (Magleby and Stevens, 1972*a*, *b*; Gage et al., 1975). Suppose for instance that there is a "closed" state (1), transition state (2), and an "open" state (3), with Helmholtz free energies of $U_1 < U_2 > U_3$. We note that if the open state, and only this state, has a dipole moment and if the dipole moment is the same for all bound agonists, then only U_3 would depend on the field and the rate constant for the closing transition would vary with voltage to the same extent for all agonists; but neither the opening rate for agonists nor the equilibrium dissociation constant for curare would be sensitive to voltage, in agreement with the data. We have argued, from the high values of k_{+2} and the even higher inferred values of k_{+1}, that these structural transitions occur as the agonist binds and dissociates from a site on the receptor protein. Thus, at the binding site the forward binding rate of agonists and antagonists depends on interionic coulomb forces; but the dissociation rate of agonists alone is modulated by a field-dipole interaction.

SUMMARY OF THE SEQUENTIAL MODEL The rate-limiting step in channel opening seems to be either a bimolecular reaction between agonist and receptor

(characterized by the rate constant k_{+2}) or an isomerization whose rate depends on a more rapid, low-affinity, bimolecular equilibrium. This opening rate has little or no voltage-dependence. By contrast, the rate-limiting step in channel closing, k_{-2}, is a voltage-dependent, first-order process, either a dissociation of the agonist or an intramolecular isomerization. Furthermore, the equilibrium number of open channels is determined by these processes with the obligatory participation of another process described by $k_{\pm 1}$. This additional process also includes a binding step, but the process cannot be resolved in our experiments both because it is too fast and because it shows little or no conductance change. Thus we have only indirect evidence for it.

COMPARISON WITH FROG ENDPLATE RECEPTORS In this paper we have reconstructed the equilibrium behavior of the channel population on the basis of kinetic measurements. Similar analyses have been applied to frog endplates (Dionne and Stevens, 1975; Neher and Sakmann, 1975; Adams, personal communication). Such studies, like the present one, reinforce the idea that channel opening and closing are governed by first-order rate constants which reflect rate-limiting molecular steps (del Castillo and Katz, 1957*a*; Steinbach, 1968; Magleby and Stevens, 1972*a*, *b*; Kordas, 1972*a*, *b*). These steps appear to take place as the last in a sequence of agonist molecules (probably two) bind to the receptor. The closing rates are well known from measurements on "noise," voltage-jump relaxations, and endplate currents (see Introduction); they agree, in actual magnitude and voltage-sensitivity, with those measured here. Opening rates have not yet been measured absolutely with experiments of the type described here but it may be inferred that they have little (Dionne and Stevens, 1975) or no (Neher and Sakmann, 1975) voltage sensitivity, in agreement with the present results on eel electroplaques.

Antagonists

Experiments at the frog endplate have ruled out effects of α-bungarotoxin and *d*TC on γ or k_{-2} (Katz and Miledi, 1972, 1973; Anderson and Stevens, 1973). The present experiments support these findings and disclose that, of the remaining possibilities, α-bungarotoxin affects only the number of available receptors, r.

Curare, on the other hand, appears to reduce the reciprocal relaxation times as well as the equilibrium conductances. Quantitatively, we have been able to fit all of our curare data merely by decreasing the forward rate k_{+2} in Eq. (11–14), with no change in the maximum conductance $r\gamma$. For instance, a concentration of 0.2 μM *d*TC—the apparent dissociation constant (Lester et al., 1975)—is fit by a twofold reduction in k_{+2}. Since this decrease applies uniformly to the receptor population it seems unlikely that when a *d*TC molecule binds to a given receptor the result is a decreased microscopic rate constant for the second binding. Rather, we interpret the *d*TC data on the basis that curare competes for an agonist binding site and that it binds and dissociates several times more rapidly than does the agonist itself. If *d*TC binding proceeded with rate constants comparable to those in Table III it would produce relaxations with several exponential components; if it proceeded much more slowly, the kinetics would be unchanged on a millisecond time scale, as seen with α-bungarotoxin. Again for 0.2 μM *d*TC, our methods would have detected an additional *d*TC relaxation

if the curare-receptor equilibrium proceeded with a time constant of about 500 μs or more. Simple relaxation theory therefore implies that the *d*TC lifetime on the receptors is no greater than 1 ms, and that *d*TC binds to receptors with a forward rate constant of at least 5×10^9 M^{-1} s^{-1}. This value is surprisingly high but apparently within the realm of possibility (Burgen, 1966).

If curarization is caused by a rapid inhibitory binding, we must explain how several seconds are required for the onset and disappearance of inhibition from iontophoretically applied pulses of *d*TC (del Castillo and Katz, 1957*b*; Waud, 1967). We suggest that such slow phenomena do not directly measure the microscopic binding rates of *d*TC, but rather reflect buffering of diffusion by multiple binding of *d*TC to receptors (Katz and Miledi, 1973; Magleby and Terrar, 1975; Adams, 1975*b*). If one ignores differences in diffusion coefficients, multiple bindings will render a molecule's rate of exit from the synaptic cleft proportional to its dissociation constant for the receptor (Colquhoun, 1975). At −90 mV curare's dissociation constant (0.2 μM) is 150 times less than that of ACh (30 μM, Table III). Thus if ACh is retained in the cleft for an extra 10 ms because multiple binding to receptors hinders its free diffusion (Katz and Miledi, 1973; Magleby and Terrar, 1975), the same mechanism would maintain the *d*TC concentration in the cleft for about 1.5 s even if, as we have suggested, *d*TC binds and dissociates from receptors on a submillisecond time scale.

The Response to a Quantum of ACh

The present data account for the rapid opening of receptor channels during the response to a quantum of ACh liberated from the presynaptic terminal. The following picture has emerged from recent studies.

(i) A quantum of ACh apparently activates more than half of the receptors reached by transmitter during the growth phase of the miniature endplate current (Hartzell et al., 1975).

(ii) The growth phase lasts about 250 μs both at nerve-muscle and at nerve-electroplaque synapses (Gage and McBurney, 1975; Lester et al., 1976).

(iii) During this growth phase the local ACh concentration is at least 300 μM (Kuffler and Yoshikami, 1975) to 1,000 μM (Fertuck and Salpeter, 1976). We have not bath applied such high ACh concentrations to esterase-inhibited preparations because rapid desensitization would result. However, the sequential model (scheme 11) predicts that opening rates continue to increase linearly with ACh; the observed constant of proportionality k_{+2} is 1 ms^{-1} for each 100 μM of ACh. Thus if a pool of receptors were subject to more than 300 μM ACh for 250 μs, more than half the channels would open.

Extrasynaptic Receptors?

On the innervated membrane of *Electrophorus* electroplaques, synaptic and extrasynaptic regions bind roughly equal total numbers of tritiated α-toxin molecules (Bourgeois et al., 1972). Frog extrasynaptic receptors have a threefold longer channel duration than do synaptic receptors (Katz and Miledi, 1972; Neher and Sakmann, 1975, 1976*b*). If electric eel receptors share this characteristic, one expects relaxations which are the superposition of two exponentials. For any model the two rate constants would differ most at low agonist concentra-

tions, where the closing rate becomes the dominant term in $1/\tau$ (e.g., $2k_{-2}$ in Eq. [13]); however, these small currents present the most difficulties for quantitative analysis. Thus we cannot decide whether the channel population has a dispersion of closing rates. Relaxations with two exponentials were sometimes observed with Sub. Nonetheless, since such results were not generally seen, we suggest that the channel population has a unique opening rate for a given agonist concentration. In terms of the sequential models all receptors have the same value for k_{+2}.

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