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Relaxation measurements on the acetylcholine receptor

(synaptic transmission/voltage clamp/ion channels/*Electrophorus* electroplaque)

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ABSTRACT In *Electrophorus* electroplaques, the agonist-induced postsynaptic conductance depends on membrane potential. During steady exposure to agonists, after a voltage step the conductance relaxes on a millisecond time scale, exponentially approaching a new equilibrium value. The relaxation rate constant k is an instantaneous function of voltage, insensitive to the past or present conductance.

Two components sum to form k. A *concentration-sensitive component* increases linearly with agonist concentration and decreases during desensitization or exposure to curare. Thus this component reflects the average frequency at which acetylcholine receptors are *opening*.

The *voltage-sensitive component*, obtained by extrapolating k to zero agonist concentration, increases at more positive potentials. For acetylcholine, the voltage-sensitive component equals the rate constant for the exponential decay of postsynaptic currents; it thus seems to be the *closing* rate for active receptors. The voltage-sensitive component has the relative amplitudes acetylcholine < carbamoylcholine < decamethonium, and for each agonist equals the closing rate determined from "noise" measurements at neuromuscular junctions.

The *kinetic* data explain several aspects of the *steady-state* conductance induced by agonists, but shed no light on apparent cooperative effects.

At a nicotinic synapse, when an agonist reaches the postsynaptic membrane, the acetylcholine receptors produce an increase in membrane conductance. In the presence of a steady agonist concentration, the receptors form an equilibrium population. At a given time, some receptors are in an "open" state (or possibly in one of two such states); other receptors are in one or more "closed" states (1, 2).

It is not yet certain which molecular events (binding of agonist, conformational change in the receptor protein, etc.) govern the transitions between the active and inactive states. In order to gain more information on these rate-limiting steps, we have applied two relaxation techniques to the acetylcholine receptors of eel electroplaques. "Voltage-jump" experiments are based on the fact that, for a steady agonist concentration, the equilibrium between "open" and "closed" receptors also depends upon the potential across the postsynaptic membrane (2–4). The proportion of open receptors increases as the potential becomes more negative. Thus, when one rapidly changes the membrane potential from one level to another, the agonist-induced conductance relaxes to a new value, with a time course which reflects the kinetics of the transition is between "open" and "closed" receptors.

The second experiment derives from the observation that, at the frog neuromuscular junction, endplate currents ordinarily last much longer than the acetylcholine concentration in the synaptic cleft (5–8). Thus the declining phase of the endplate current is the response of receptors to an instantaneous removal of acetylcholine. We report here that the declining phase of eel postsynaptic currents has precisely the same significance.

We find that the kinetic data account qualitatively for the behavior of the steady-state conductance, as influenced by agonist concentration and by membrane voltage. In addition, the steady-state conductance seems governed by cooperative effects whose molecular nature remains unexplained.

MATERIALS AND METHODS

The experiments use the isolated electroplaque from the organ of Sachs of *Electrophorus electricus*. The innervated face is held against a Mylar sheet which partitions two pools of Ringer's solution; the sheet contains a window that exposes part of the innervated face to one of the pools (9). Currents are passed between platinum plates in each pool, traversing the window and the exposed region of the innervated face (4, 10). The temperature was 15° unless otherwise noted.

The voltage-clamp procedure was used because conductances may be measured at controlled voltages and with temporal resolution which is dictated by the speed of a feedback circuit rather than by the time constant of the membrane. The feedback circuit was connected to the preparation only during the actual clamping episodes; a trial consisted of 10 to 20 such episodes spaced 300–1000 msec apart (see Fig. 1). Thus, although agonists were added for several minutes at a time during "voltage-jump" experiments, there was usually no external current applied to the cell.

For measurements of postsynaptic currents, at the start of each episode the potential was clamped at +50 mV for several msec to inactivate Na conductance in the electroplaque. Next a strong, brief (200 μsec) pulse of current was passed so as to depolarize the innervated face; this evoked a spike in the motor nerve terminals. Then the voltage was clamped at the desired level for measuring the postsynaptic current.

The Ringer's solution contained Ba^{+2} (3 mM), which eliminates the inward rectifying conductance for K^+ (4, 11), and, for "voltage-jump" experiments, tetrodotoxin (10^{-6} M), which eliminates Na activation. The electroplaque does not show delayed rectification for K^+. Therefore, in the absence of agonists, the innervated membrane showed a linear current-voltage (I–V) plot (4, 11).

For "voltage-jump" experiments with acetylcholine, and occasionally with other agonists, cells were pretreated with diisopropyl fluorophosphate (4×10^{-4} M) for 30 min at 23° in Ringer's solution followed by two 20-min washes in Ringer's solution. This treatment is known to inhibit more than 90% of the acetylcholinesterase (12) but had no effect on the acetylcholine receptor, as judged by the response to carbamoylcholine.

Other details of the experiments and methods of analysis have been given (4).

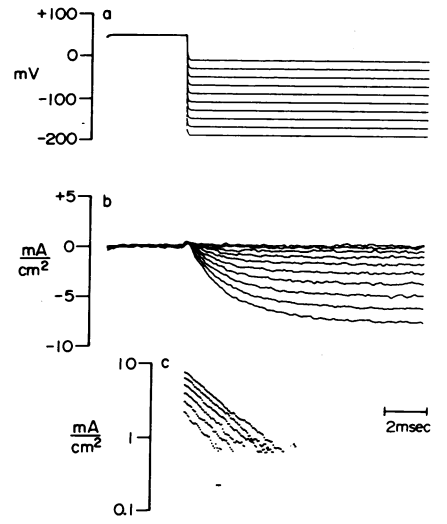

FIG. 1. Data from a voltage-clamp trial with acetylcholine, 25 μM. Time axis applies to all three panels. (a) Superimposed traces of membrane voltage. Second voltage level is changed for each episode. (b) Agonist-induced currents; leakage and capacitative currents have been subtracted. (c) Data for the lower six traces in (b). Semilogarithmic plot of the approach to steady state. Points start 180 μsec after the jump.

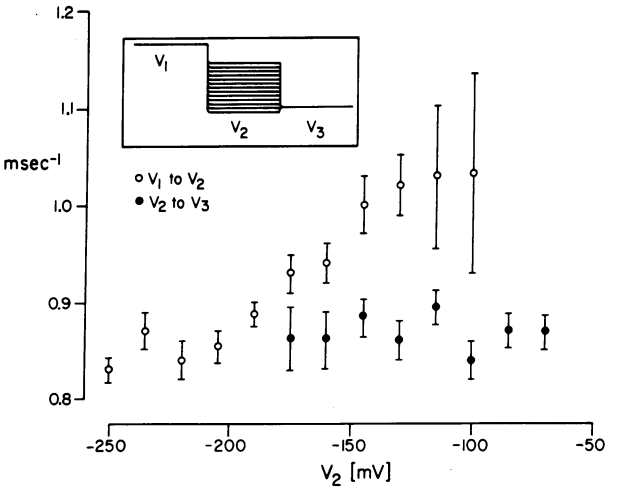

FIG. 2. Evidence that k depends instantaneously on V. Carbamoylcholine, 100 μM. Superimposed voltage traces are shown in the inset; V_2 lasted 7.5 msec. V_1 = +50 mV; V_3 = −235 mV. Ordinate, relaxation rates (k) for the agonist-induced *currents*. o, data for jump from V_1 to V_2; •, V_2 to V_3. 95% confidence limits are shown. The jump from V_2 to V_3 also produced instanteous ohmic changes in agonist-induced currents, due to existing open receptors.

RESULTS

Voltage-jump experiments

Fig. 1 shows records from an experiment designed to measure the voltage-controlled increase of conductance in the presence of 25 μM acetylcholine. The receptors were first forced into a closed state by holding the voltage at +50 mV for 4 msec; the voltage was then jumped to various more negative values. Agonist-induced currents increased after the jump, along an exponential time course (Fig. 1c).

The rate constants (k) of this relaxation, as defined by the slope of the lines in Fig. 1c, are instantaneous functions of the potential. This conclusion is derived from experiments in which the initial and final levels of voltage jumps were independently varied; k depended only on the final voltage (Fig. 2). Thus there is no detectable dependence of k upon the conductance, the history of the conductance, or the history of the potential.

The rate constants also increase with increasing agonist concentration (Fig. 3). For a given cell, this relation seems linear (Fig. 4), and the slope does not vary with potential. For zero agonist concentrations the linear trend extrapolates to a finite k; this intercept varies with potential. Each agonist may be characterized by the slope and intercept of plots of k against concentration such as Fig. 4 (Table 1).

With high agonist concentrations (e.g., 250 μM carbamoylcholine, 100 μM acetylcholine, 10 μM decamethonium), the conductances and relaxation rates undergo substantial desensitization (Fig. 5), although less rapidly at 15° than

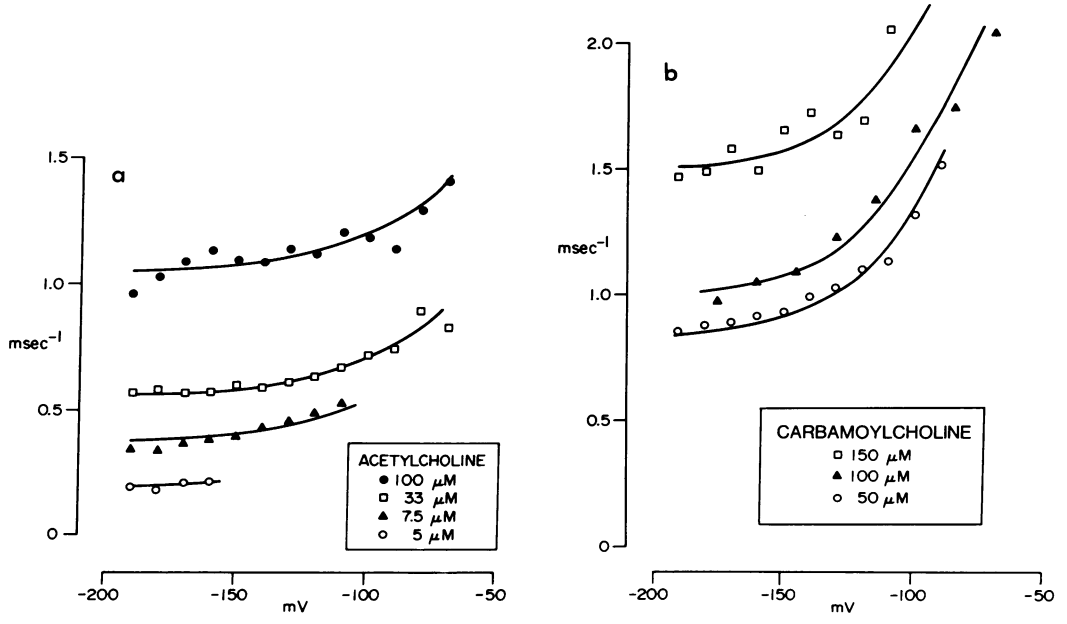

FIG. 3. Relaxation rate constants (k) at several voltages and agonist concentrations; trials like that of Fig. 1. Ordinate, k; abscissa, final V. Data from several cells.

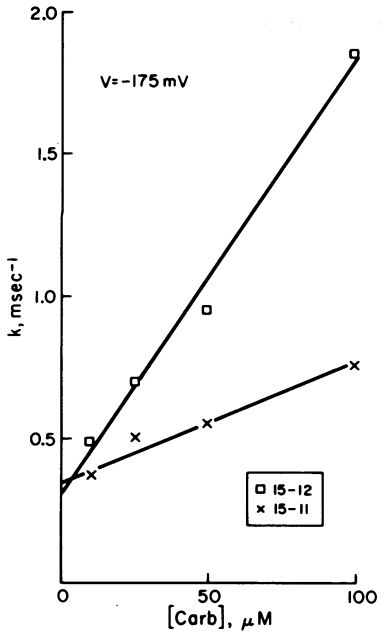

FIG. 4. Concentration dependence of relaxation rate constant (k). Two cells from the same eel, tested on the same day.

at 22° (4). To measure approximately the response that would occur in the hypothetical absence of desensitization, we have taken the rates at their peak with time (4). For carbamoylcholine and acetylcholine, the linear increase of k extended to these desensitizing concentrations. For decamethonium, the peak rates often appeared to level off at concentrations between 10 and 20 μM.

Steady-state conductances induced by agonists are competitively inhibited by d-tubocurarine, with an apparent dissociation constant of 0.2 μM (4). The relaxation rates k are also reduced by d-tubocurarine. For one cell tested with carbamoylcholine, the effect of 0.2 μM d-tubocurarine could be described as a decrease by 40% in the slope of the plot of k against concentration. The intercept changed by less than 10%.

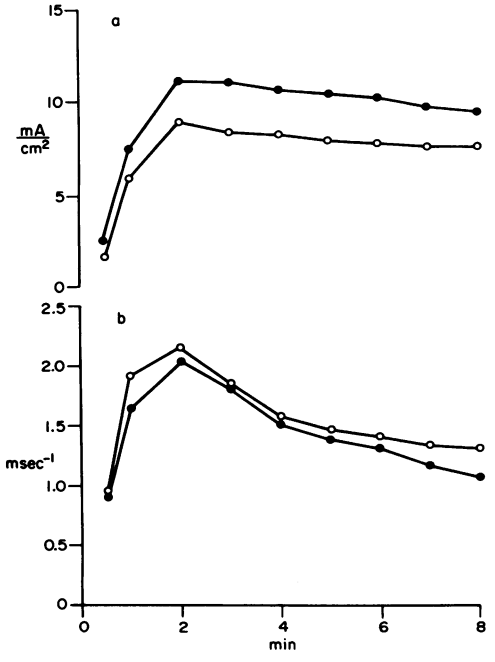

FIG. 5. Desensitization. Trials like that of Fig. 1. Carbamoylcholine (250 μM) was introduced at time zero. (a) Steady-state agonist-induced currents. (Sign convention is opposite to that of Fig. 1.) (b) Relaxation rates, k. ○, −130 mV; ●, −160 mV.

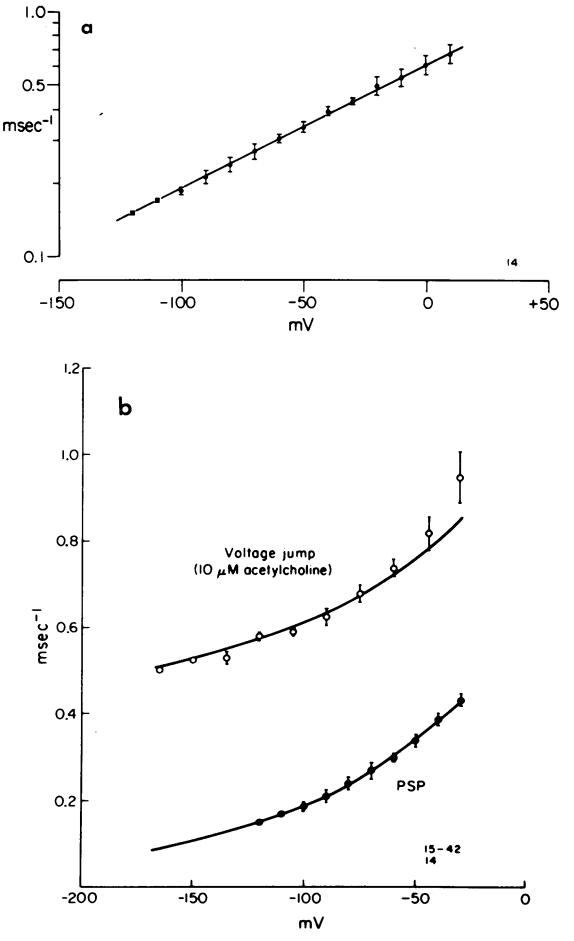

FIG. 6. (a) Decay rate of postsynaptic currents against voltage; average (±2 SEM) of five different cells. (b) Comparison between data in (a) and a cell which gave the most complete "voltage jump" data for acetylcholine (pretreated with diisopropyl fluorophosphate; see *Materials and Methods*). Error bars show two SEM, if larger than symbol. The solid line through the postsynaptic current (PSP) data is the exponential relation from (a); the same line, plus a constant, has been drawn through the "voltage jump" data. Note that ordinate is logarithmic in (a), linear in (b).

The steady-state conductances were reduced irreversibly by a factor of two to three after exposure to 50 nM α-bungarotoxin for 15 min; the relaxation rate constants, however, remained unchanged.

The rate constants vary with temperature. At 15° and −150 mV, k for decamethonium (3 μM) was 1.08 msec^{-1}; at

Table 1.

Agonist	Slope (M^{-1} sec^{-1})	Intercept ($msec^{-1}$)
Acetylcholine	10^7	0.1
Carbamoylcholine	10^7	0.3
Decamethonium	2×10^8	0.5

Intercept (at zero concentration, −175 mV) and slope of plots of k against concentration like Fig. 4. Slopes have an SEM of 50%; intercepts, 25%.

25° and the same potential, k increased to 3.47 $msec^{-1}$. This change corresponds to a Q_{10} of 3.2. In contrast, the steady-state conductance induced by a given agonist varies little with temperature; the Q_{10} was less than 1.5.

Postsynaptic currents

Postsynaptic currents decay with a single exponential rate constant. The rate constant increases at more positive potentials. At 15° the rate constant equals 0.65 $msec^{-1}$ at 0 mV and changes e-fold with each 85 mV (Fig. 6a). At 22° these values increase to 2.1 $msec^{-1}$ and 121 mV. All these data agree closely with those for frog endplate currents (5, 6).

The voltage sensitivity of postsynaptic current decay rates is very similar to that of the relaxation rates (k) for acetylcholine (Fig. 6b). The "voltage-jump" rates nevertheless have a slightly but consistently larger voltage-sensitive component than do the decay rates for postsynaptic currents (Fig. 6b is an example); however, these differences vanish when "voltage-jumps" for a given temperature are compared with postsynaptic currents measured at about 1° higher. To explain these facts, we note that "voltage-jump" relaxations are measured soon after the agonist has been introduced by a rapid solution change. Small temperature increases occur near the electroplaque during such changes; because of the chamber's geometry, these increases are probably not recorded accurately by the thermistor.

DISCUSSION

If the receptor has a unique "open" state, voltage-clamp currents provide, for a given potential, a linear measure of the number of open receptors. Over a wide range of conditions, we have found that currents relax with a single rate constant after a voltage jump. A simple exponential also characterizes the decay phase of postsynaptic currents.

Separation of "opening" and "closing" rates

A rather general physical model to account for the data is that the number, r_a, of open receptors depends upon two first-order unidirectional rate constants: the "opening" rate δ and the "closing" rate α. One or both of these rates depends on voltage. Then, if r is the total number of receptors,

$$dr_a/dt = -\alpha r_a + \delta(r - r_a) \qquad [1a]$$

The rate constant k for relaxtion after a voltage jump is

$$k = \alpha + \delta \qquad [1b]$$

and the equilibrium number of open receptors is $r\delta/(\delta + \alpha)$.

During the declining phase of postsynaptic currents, δ is zero (5, 6). Hence for acetylcholine, α is given by the rate of decline of postsynaptic currents. We find that

$$\alpha = \alpha_0 \exp(V/V_1), \qquad [2]$$

where α_0 is 0.65 $msec^{-1}$ and V_1 is 85 mV at 15°. For acetylcholine receptors at the frog endplate, these same values of α_0 and V_1 have been obtained in studies of each of three phenomena: (*i*) the decay of endplate currents (5, 6); (*ii*) acetylcholine-induced "noise" (2); and (*iii*) "voltage-jump" relaxations at low acetylcholine concentrations (13, 14).

In the present "voltage-jump" experiments, the relaxation rate k increases with higher agonist concentration and decreases during desensitization and curare treatment. Such manipulations affect, of course, the steady-state number of open receptors, r_a, but apparently alter neither the conductance γ nor the lifetime $1/\alpha$ of the open state (1, 2). Thus, these changes in k seem to reflect changes in the opening rate δ.

Since $\delta = k - \alpha$, we find for acetylcholine that δ does not vary detectably with potential (Fig. 6b). Also, δ seems to increase in a roughly linear fashion with agonist concentration.

Postsynaptic currents give a measure of α for only one agonist, acetylcholine. However, effects of carbamoylcholine, decamethonium, and acetylcholine can be compared with the technique of bath application. For small agonist-induced conductances, all three agonists conform to the same pattern, in the sense that they all produce an *equal* exponential voltage-dependence of the equilibrium conductance (ref. 4 and the present experiments). Since small conductances are determined by the ratio δ/α, we suggest that δ and α behave similarly for all agonists. Thus, δ would be voltage-independent, dependent on the agonist and probably proportional to its concentration; α would be given by equation (2) where α_0 varies with the nature of the agonist but V_1 does not and equals 85 mV at 15°. At any potential α is also extrapolated zero-concentration value of k (Fig. 4); data for −175 mV are given in Table 1.

The relaxation rates k are well described by this scheme and by Eqs. **1b** and **2** (see upper panel in Fig. 7), with α_0 = 2.0 $msec^{-1}$ (range 1.8–2.2) for carbamoylcholine and 3.1 $msec^{-1}$ (range 3.0–3.4) for decamethonium. The receptor opens for an average time $1/\alpha$; at all potentials this time is in the order, acetylcholine > carbamoylcholine > decamethonium, in agreement with "noise" data (14, 16).

Prediction of equilibrium data

The open receptor has a linear I–V relation (4). Then if γ is the open conductance and V_0 the reversal potential, Eq. 1 leads to the steady-state I–V relation:

$$I = r\gamma[\delta/(\alpha + \delta)](V - V_0). \qquad [3]$$

This theory does not fit the data, even if δ, α_0, and V_1 are a factor of two different from the measured values. However, an excellent fit is obtained for acetylcholine and carbamoylcholine with the relation

$$I = r\gamma[\delta/(\alpha + \delta)]^n (V - V_0) \qquad [4]$$

where n is the Hill coefficient of the initial part of the dose-response curve (4); n averages 1.9 and lies between 1.5 and 2 for different cells (Fig. 7). The average value for $r\gamma$ is 0.11 mho/cm^2 (range 0.07–0.17).* The data are described well by a linear dependence of δ on agonist concentration. For a given cell, the effect of *d*-tubocurarine or of desensitization is simply to decrease δ; that of α-bungarotoxin, to decrease $r\gamma$ [probably by decreasing r (15)].

These findings do not uniquely describe the molecular nature of receptor activation, although the mechanism is evidently a cooperative one at some level. We are particularly puzzled that although some cooperative and multistate models (17, 18) give an equilibrium expression similar to that of Eq. 4, each predicts that either voltage-jump relaxations or endplate current decays, or both, would depart measurably from the simple exponentials we have observed.

* For decamethonium, the rate constants are not sufficiently precise to give a reliable estimate for n or $r\gamma$.

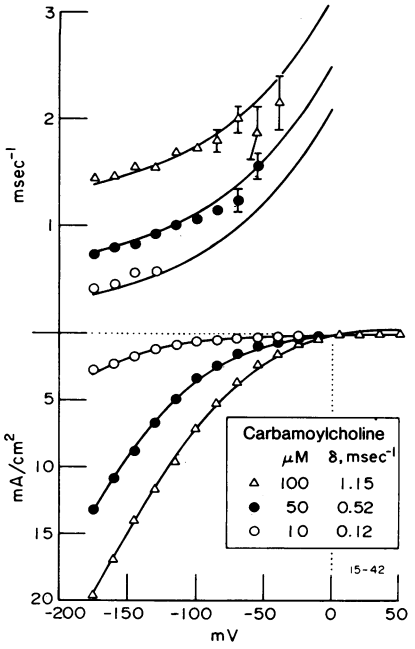

FIG. 7. Comparison of Eqs. 1b, 2, and 4 (lines) with experimental data for one cell. Top, relaxation rate k. Bottom, agonist-induced currents at steady state (i.e., vertical displacement of traces at extreme right in Fig. 1b). Assumed values of δ are given. Other parameters are $n = 2$, $\alpha_0 = 2$ msec^{-1}, $V_1 = -85$ mV, and $r\gamma = 0.17$ mho/cm^2.

Rates and equilibria at the receptor

Neglecting the problems posed by cooperativity, we briefly examine two molecular hypotheses:

If binding of agonist is the rate-limiting step in activation of the postsynaptic conductance,

$$\text{agonist} + \text{binding site} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{agonist-binding site},$$

then assuming that the agonist concentration near the receptors is buffered by the bulk solution (19), $\delta = k_{12}$·[agonist]. Thus the forward rate k_{12} is the slope of the dose-response line in Fig. 4 (see Table 1). Furthermore, α equals k_{21} and (at a given voltage) is the intercept of the same line (Table 1). The dissociation constant K_D is given by k_{21}/k_{12}. In this model k_{21} and K_D decrease at more negative potentials, at a rate of e-fold for each 85 mV. At zero mV, K_D is 65 μM for acetylcholine, 200 μM for carbamoylcholine, and 15 μM for decamethonium. Desensitization and exposure to d-tubocurarine decrease the forward rate k_{12}.

The alternative hypothesis, namely that transitions or polymerizations (18) of the channel or "ionophore" are the rate-limiting steps measured in these relaxations, would imply that the closing rate of the ionophore, α, is "induced" differently by each agonist and would demand that δ level off at high agonist concentrations. Such leveling off has been seen for decamethonium, but rapid desensitization may vitiate accurate measurements in the relevant range of agonist concentrations (4).

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