Kinetic constants of the acetylcholine (ACh) receptor reaction deduced from the rise in open probability after steps in ACh concentration

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ABSTRACT Outside-out patches of enzymatically dissociated adult and denervated mouse muscle fibers were superfused repetitively by pulses of acetylcholine (ACh) containing solution. Up to 300 channels opened simultaneously 300 μs after the beginning of a 1,000 μM ACh pulse corresponding to a peak current î of almost −1 nA. Single responses to ACh were averaged and the concentration dependence of î and of the rise time t_r from 0.1 î to 0.9 î was measured. In adult receptors, î increased proportional to the second to third power of ACh concentration, whereas in embryonic-type receptors it was proportional to the first to the second power. t_r increased from ~0.3 ms at 1,000 μM ACh to a plateau value of ~5 ms for adult and of ~10 ms for embryoniclike receptors at concentrations <10 μM ACh. The concentration dependence of î and t_r was simulated using the standard model of ACh binding with different combinations of rate constants and two and three binding sites for ACh. The calculated curves were compared to the measurements and a set of well fitting rate constants was determined for adult and embryoniclike receptors. Three binding sites for ACh were necessary to fit the dose response for î for adult receptors. A method for deriving rate constants in a model of ACh-receptor interaction is described that avoids analysis of open-closed kinetics of single channels, which in rapid systems, as the ones studied here, are at the limit of the frequency response of the current measurement.

INTRODUCTION

The patterns of opening of nicotinic receptor-channel complexes have been studied most in the steady state. Usually, cell-attached patch-clamp recordings were carried out with a fixed acetylcholine (ACh) concentration contained in the pipette (e.g., Hamill et al., 1981; Hamill and Sakmann, 1981; Dionne and Leibowitz, 1982; Jackson et al., 1982; Auerbach and Sachs, 1984; Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986; Jaramillo and Schuetze, 1988). These studies resulted in detailed models of the activation reactions which were based on the conclusions of del Castillo and Katz (1957) and were extended, e.g., by Magleby and Stevens (1972), Colquhoun and Hawkes (1977), Dreyer et al. (1978), Dionne et al. (1978), Colquhoun and Hawkes (1982), and Colquhoun and Sakmann (1985). The choice of models and the estimation of parameters depended critically on the measurement of very short openings and closings of the channels, which are at the limits or beyond the limit of possible frequency resolution. The forward rates of receptor activation cannot be measured directly at all.

The reaction kinetics estimated from the steady-state effects of a given ACh concentration should also predict the dynamic channel behavior in response to changes of ACh concentration, as observed in synaptic transmission or in pulsed applications of ACh. The measurement of average transients of channel opening after pulses of ACh is less limited by problems of frequency resolution than the recording of single-channel currents. On the other hand, these transients may contain enough information to deduce kinetic constants. We have developed a method of rapid application of agonists to excised patches (Franke et al., 1987; Dudel et al., 1990). Pulses of ACh to patches from adult or denervated muscles of mice elicit rapidly rising and desensitizing channel activation (Franke et al., 1991*a*, *b*). The rise times and peaks of such responses can be determined for different concentrations of ACh. The models were evaluated by simulating the respective set of differential equations, and their numerical solution by computer. Colquhoun and Hawkes (1977) have discussed similar models with probabilistic equations, assuming Markov-chain processes. They give solutions for steps in agonist concentration in matrix form. Solution of the matrices yields a sum of three exponentials, the terms of the exponents filling more than one page in print. Alternative to the differential equations used here, such matrices could have been evaluated numerically, presumably giving equivalent results.

METHODS

Interosseal muscles from the hind feet of adult mice were dissociated enzymatically by superfusion with a solution containing 0.5 mg/ml Type Ia collagenase (Sigma Chemical Co., St. Louis, MO). They were stored in culture dishes. In the first 10 h after dissociation of the muscles, the ACh receptors were considered to be of the mature, "adult" type (Franke et al., 1991*a*). During dissociation, the presynaptic nerve terminals were removed. When the dissociated muscle fibers

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were kept in tissue cultures for 14 d, extrasynaptic ACh receptors changed to the "denervated," embryoniclike type (Franke et al., 1991*b*). For the measurements, the muscle fibers in the culture dishes were superfused with physiological solution containing 162 mM NaCl, 5.3 mM KCl, 2 mM $CaCl_2$, 0.67 mM NaH_2PO_4, 15 mM HEPES buffer, 5.6 mM glucose; the pH was 7.4. The preparations were held at room temperature (21°C) and could be used for patch-clamp measurements for 1–2 h. Outside-out patches were prepared as described by Hamill et al. (1981). The solution inside the patch pipette contained 140 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES buffer, and 10 mM glucose; pH 7.2. In adult preparations, patches taken from the endplate region or from its vicinity yielded identical results. In "denervated" preparations, patches were obtained from the extrasynaptic membrane.

The patch pipette containing the outside-out patch of muscle membrane was placed into a liquid filament switch in which the jet of solution hitting the patch could be shifted from control solution to a solution containing a defined ACh concentration within 0.2 ms (Franke et al., 1987; Dudel et al., 1990). Channel currents were measured with a Neher-Sigworth (EPC7) or a Dagan 3900 amplifier (List, Darmstadt, Germany, and Dajan, Madison, WI, respectively). The records were stored on a video tape (modified Sony PCM-502 ES). For off-line analysis, the data were digitized at 20–50 kHz and evaluated on a series 300 microcomputer (Hewlett-Packard, Palo Alto, CA) (Franke et al., 1986; Dudel and Franke, 1987).

The simulation of the reaction kinetics was based on the relevant parts of a program employed in Parnas et al. (1989), which was extended to allow for maintained ACh concentrations and three binding step reactions after the ACh concentration step. The simulations were run on IBM AT personal computers.

RESULTS

Responses of adult muscle receptors

Original single patch-clamp recordings from a patch excised from adult mouse muscle are shown in Fig. 1 (*left column*). ACh applied for 500 ms at low concentrations elicited isolated or superimposed single-channel openings. These openings have a conductance $\gamma = 65$ pS and average open times of ~1 ms (Franke et al., 1991*a*, *b*). When the ACh concentration was raised, peak currents increased (note the different scales) whereas desensitization became more and more rapid and extensive. The concentration dependence of desensitization is discussed in Franke et al. (1991*a*) and this process will be disregarded in the present study. With 1,000 μM ACh, the peak current was −486 pA, which corresponds to the

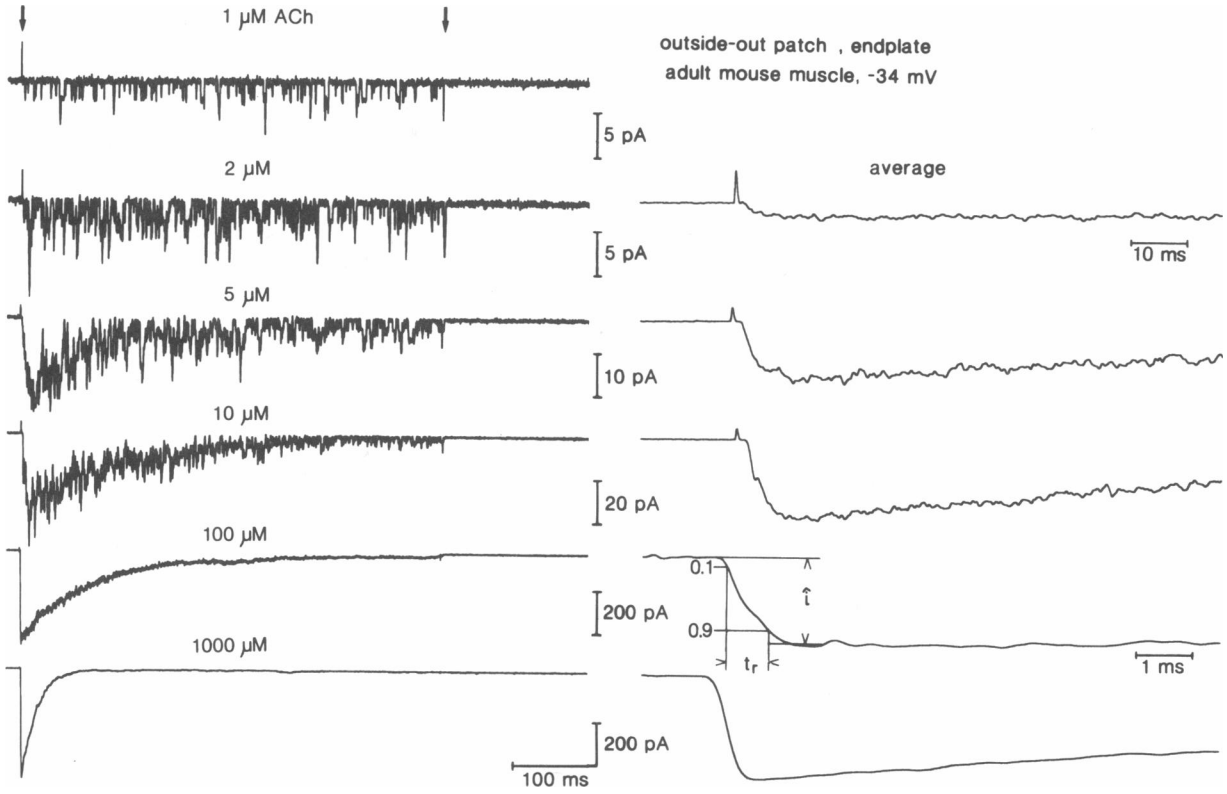

FIGURE 1 Activation of nAChR channels on an outside-out patch from the end plate of an adult muscle fiber by 500-ms ACh pulses, membrane potential −34 mV. (Left column): single responses to pulses with 6 ACh concentrations. Note different current scales. (Right column): averages of single responses as shown on the left hand side. 212, 81, 23, 6, and 4 tracks were averaged at 2, 5, 10, 100, and 1,000 μM, respectively. The evaluation of the peak current î and of the rise time t_r from 0.1 to 0.9 î is indicated.

simultaneous opening of ~243 channels. In frog muscle, 1 mM ACh opened channels with a probability of 0.95 (Franke et al., 1991*a*). Such high open probabilities are likely to apply for mouse muscle as well. The number of channels present in the patch is estimated to be ~250.

Summation of single recordings generated by ACh pulses with the same concentration yielded the average currents in Fig. 1 (*right column*). The time scales were expanded for evaluation of the rise time t_r. At ACh-concentrations of 100 μM, the rise times were longer than 0.8 ms and were probably not seriously distorted by the rise time of the ACh concentration of ~0.2 ms (Dudel et al., 1990). With 1,000 μM ACh, the rise time was at ~0.5 ms and this value is probably too large as a result of the noninstantaneous rise in ACh concentration. The rise time with 1,000 μM ACh may therefore be taken only as an upper limit of the true value.

The evaluation of the peak currents, î, and of the rise times, t_r, from the experiment of Fig. 1 and from two other experiments are shown in Fig. 2. The î values were normalized, with respect to the î measured with 1,000 μM ACh. As discussed above, the channel open probability is assumed to be close to one at this ACh concentration, and the ordinate scales of relative peak current can be read also as approximate open probability. The dose-response curves measured for relative î were very similar in all preparations. Below 10 μM ACh, they rise with double-logarithmic slopes of 2.1–2.3. The respective slopes in Franke et al. (1991*a*) were 2.3–2.8, i.e., also >2. Above 100 μM ACh î approached the saturation level. Similar dose-response curves were seen in 12 patches.

The second graph in Fig. 2 presents dose-response curves for t_r, of the same patches for which î was determined. t_r appears to have two saturation levels, one of ~5 ms at low ACh concentrations, and one between 1 and 0.5 ms at high ACh concentrations. For two of the t_r curves, the switch from one level to the other occurred between 5 and 100 μM ACh, whereas in the third curve the change in t_r extended to lower concentrations. In spite of the relatively large differences in the t_r curves, the corresponding î curves were very similar. These differences are typical and were seen also in further patches.

Responses of receptors from denervated muscles

Specimen recordings from this channel type and average currents corresponding to Fig. 1 were shown in Franke et al. (1991*b*). The elicited currents also exhibited rapid desensitization. The single-channel currents measured at this stage of denervation were smaller (γ = 36 pS) than those of adult muscle, and the openings were longer, presenting average open times of ~2.3 ms. This agrees with descriptions of these channel types in the literature (compare Schuetze and Role, 1987).

The dose-response curves for î (Fig. 3) were less steep than those for adult muscles. The maximal double-logarithmic slope for low ACh concentrations was 1.7–2.1. The corresponding dose-response curves in Franke et al. (1991*b*) showed maximal slopes of 1.1 to 1.7. Therefore, the double-logarithmic slopes of these dose-response curves were probably ≤2. Above 10 μM ACh the dose-response curves of î approached the saturation level of 1. Sine and Steinbach (1984) described that open channel block of the agonist affects single channel currents if concentrations of 1,000 μM ACh and higher are used. In steady-state measurements with 1,000 μM ACh, we did not see rapid transitions between open and closed state of the channel (Franke et al., 1991*b*), indicating open channel block. Open channel block is voltage dependent and may be prevented in the outside-out mode by an alteration of the surface potential.

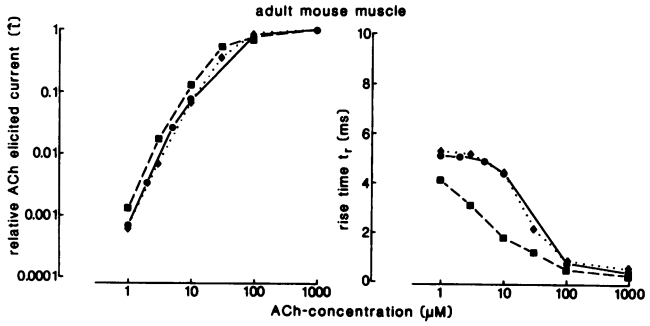

FIGURE 2 Concentration dependence of the peak current î and the rise time t_r for three adult muscle preparations. Each experiment is plotted with the same symbol and line type. î is normalized to the value of î with 1,000 μM ACh.

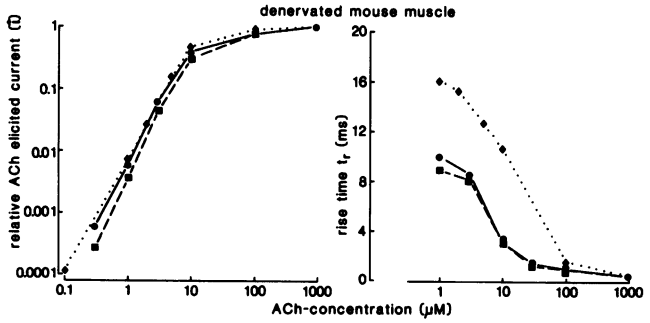

FIGURE 3 Concentration dependence of the peak current î (*left side*) and of the rise time t_r (*right side*) for three different denervated muscle preparations. Each experiment is plotted with the same symbols and line type. î is normalized to the value of î with 1,000 μM ACh.

The dose-response curves for the rise times t_r are plotted also in Fig. 3. Compared to the adult muscles, the t_r for low ACh concentrations were higher in the denervated muscles, the range was 8–16 ms. Similar to the adult muscle, these t_r values had a wide range of variation, with little corresponding variation in the î. In contrast, in all patches t_r declined to <1 ms for >100 μM ACh, with little variation in the limiting rise times.

Modeled dose-response curves for î and t_r

Variation of parameters

In the second part of this study, we shall try to compare the results of the experimental section to models of the ACh-receptor reaction, with the aim to establish a reasonably accurate description of the experimental curves. We start from the model reaction 1, in which A is an agonist, in our case ACh, R the receptor-channel, and R^* the opened receptor-channel (Parnas et al., 1989),

$$\begin{matrix} A & & A & & & & \\ + & 2k_1 & + & k_2 & & \beta & \\ R & \rightleftharpoons & AR & \rightleftharpoons & A_2R & \rightleftharpoons & A_2R^*. \\ & k_{-1} & & 2k_{-2} & & \alpha & \end{matrix} \qquad (1)$$

REACTION 1

This is a simplified version of the principal model discussed recently by Colquhoun and Sakmann (1985). The complete model of the latter study comprises further an opening reaction $AR \rightleftharpoons AR^*$ which generates short openings mainly at low ACh concentrations. Further, the complete model contains an $A + AR^* \rightleftharpoons A_2R^*$ step, which is considered to be of little importance. We shall start the analysis using the simplified reaction 1. As is discussed below, inclusion of an $AR \rightleftharpoons AR^*$ step with the rate constants proposed by Colquhoun and Sakmann (1985) has insignificant effects on the dose-response curves.

To find a suitable combination of parameters in reaction 1, the rate constants were varied systematically trying to estimate their contribution to the dose-response curves for î and t_r. The variations in each case started from 'initial values' or controls which were the same for all simulations in Figs. 4–6. These initial values were taken from Colquhoun and Sakmann (1985):

$$k_1 = k_2 = 10^8 M^{-1}s^{-1}, k_{-1} = k_{-2} = 8{,}000\ s^{-1},$$
$$\alpha = 700\ s^{-1} \text{ and } \beta = 30{,}000\ s^{-1}.$$

These parameters were estimated from the kinetic characteristics of channel openings elicited by a low ACh concentration in adult frog muscle at 11°C. In our measurements, the temperature was 21°C. Nevertheless, the initial values proved to be quite applicable. The control curves generated with these initial parameters were drawn out in the simulations of Figs. 4–6 and are presented as constant reference curves in each graph (*solid lines*).

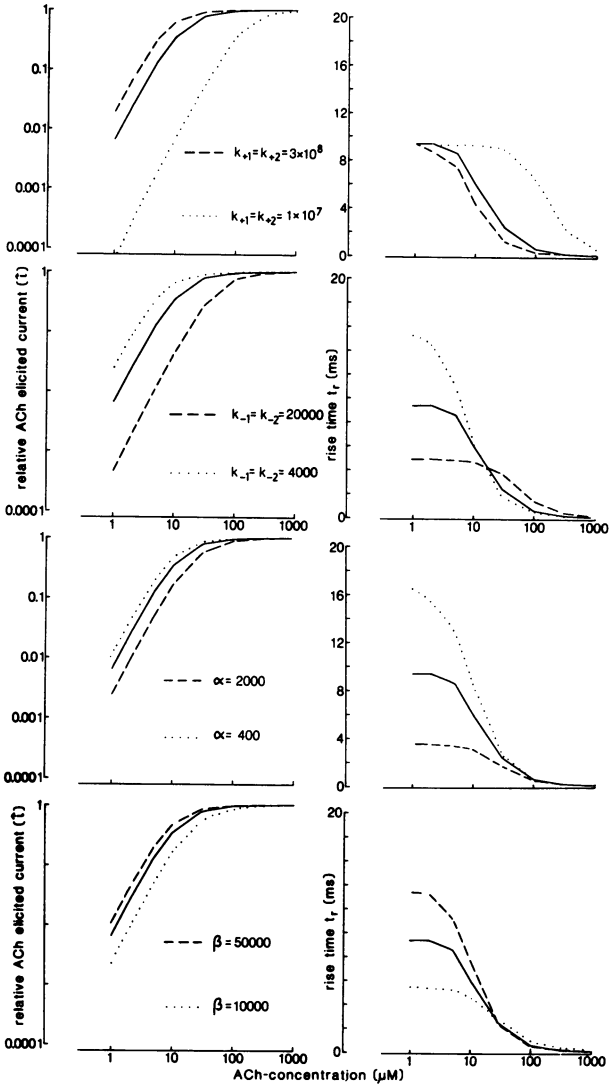

FIGURE 4 Simulations of the concentration dependence of the peak current î and of the rise time t_r calculated with different combinations of rate constants of reaction 1. î and t_r were calculated at 1, 2, 5, 10, 30, 100, 300, and 1,000 μM ACh. In each graph, the solid curve represents the simulation with the control parameters (Colquhoun and Sakmann, 1985). The two other lines correspond to two variations of the parameters as indicated in each panel.

As a first step, it was assumed that the binding and unbinding rates $k_1 = k_2$, and $k_{-1} = k_{-2}$. This reduced the parameters in reaction 1 to four. Each of them was varied in Fig. 4 by increasing the respective initial value (*dashed curves*) or by decreasing the respective initial value (*dotted curves*) to the levels indicated in the respective pairs of graphs. The amplitudes of the varia-

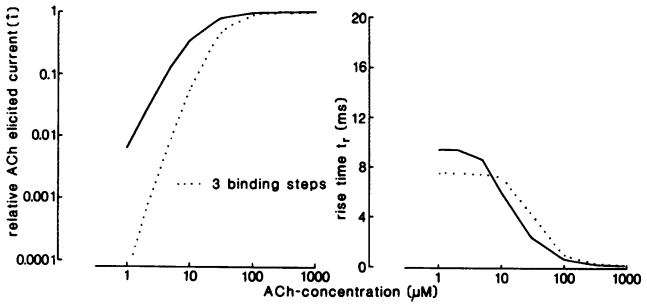

FIGURE 5 Simulations of the concentration dependence of the peak current î and the rise time t_r calculated with two (*solid line*) and three (*dashed line*) binding steps. Control parameters also for three binding steps (reaction 2), i.e., $k_{+3} = k_{+2} = k_{+1}$ and $k_{-3} = k_{-2} = k_{-1}$.

tions of the parameters were chosen to give clear-cut effects. Many intermediate values were tried, and there was no indication of inhomogeneities in the responses to the variations within the tested range.

The control curves in Fig. 4 appear already suitable to fit the measured curves (Figs. 2 and 3). The shapes and the ranges of the dose-response curves for î and for t_r were met fairly well. The binding and unbinding rates, k_1 and k_{-1} affected the dose-response curves for î relatively strongly. Reduction of k_1 to 10^7 M^{-1} s^{-1} and also increase of k_{-1} to 20,000 s^{-1} shifted this relation to the right, indicating a higher K_m value. In contrast, variation in α or β did not have much effect on the î curves. The t_r curves appeared to be more sensitive to parameter variations than the î curves. Reduction of k_1 shifted the t_r curve to the right, whereas variation of the other parameters mainly affected the plateau of t_r at low ACh concentrations. The combined reactions of î and t_r are often difficult to understand intuitively. For instance, in Fig. 4, decrease of k_{-1} or of α, and also increase of β shift

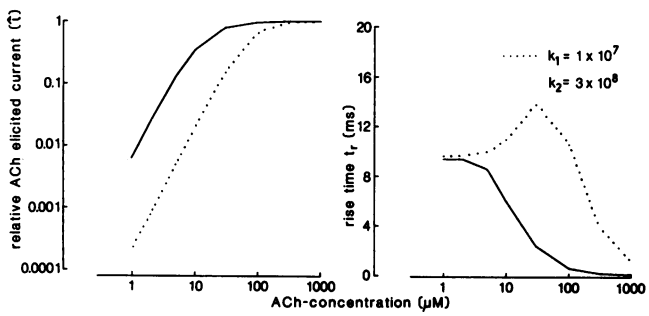

FIGURE 6 Simulations of the concentration dependence of the peak current î and rise time t_r for two binding steps and control parameters (*solid line*), and for positive cooperativity ($k_2 > k_1$, *dashed line*). The values used for k_1 and k_2 are indicated.

the î dose-response to the left, increasing the affinity. However, this increased affinity is associated with a lengthening of the rising phase t_r at low ACh concentrations. The simulations discussed so far show that the possible effects of variations of the four parameters on î and t_r were quite diverse. Sets of parameters suitable for fitting the measured relations proved to be rather unique (Fig. 7).

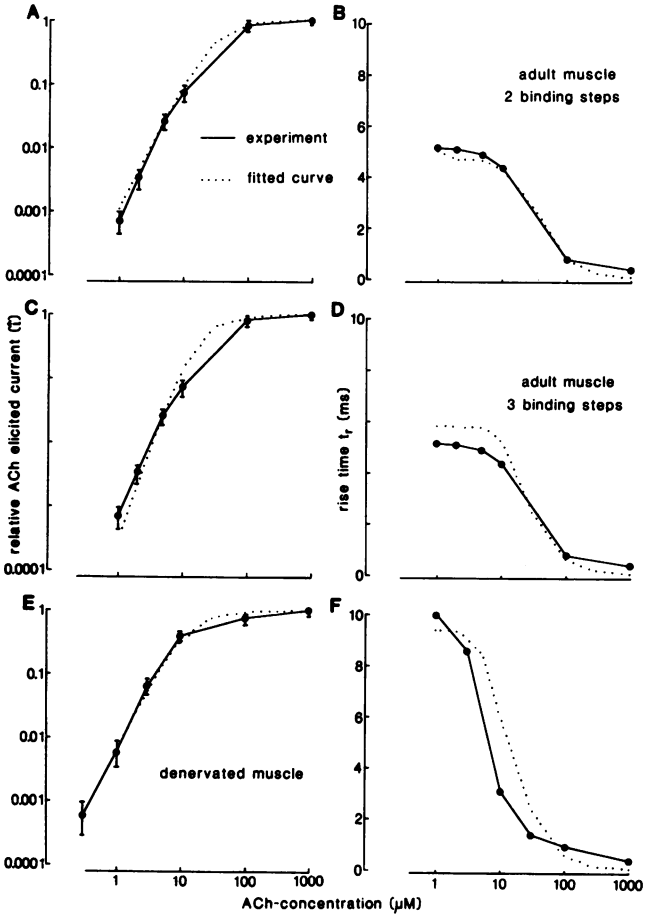

FIGURE 7 Concentration dependence of the peak current î and the rise time t_r for a typical experiment (*solid lines*) with adult (*A–D*) and with denervated (*E* and *F*) muscle. The error bars represent a standard error which was assumed to be equal to the noise in the averaged traces: $SE = i_s \cdot \sqrt{N_s/N}$, where i_s is the single-channel current, N_s the average number of open channels in the peak, and N the number of sweeps averaged. The dashed lines represent the optimal fit with two (*A* and *B*, reaction 1) and three (*C* and *D*, reaction 2) binding steps for adult muscle and with two binding steps (*E* and *F*, reaction 1) for denervated muscle. The rate constants for the optimal fit were for *A* and *B*: $k_{+1} = k_{+2} = 10^8$ M^{-1} s^{-1}, $k_{-1} = k_{-2} = 20{,}000$ s^{-1}, $\alpha = 1{,}100$ s^{-1}, $\beta = 50{,}000$ s^{-1}; for *C* and *D*: $k_{+1} = k_{+2} = k_{+3} = 10^8$ M^{-1} s^{-1}, $k_{-1} = k_{-2} = k_{-3} = 4{,}000$ s^{-1}, $\alpha = 1{,}500$ s^{-1}, $\beta = 50{,}000$ s^{-1}, and control parameters for *E* and *F*.

In further simulations we have tried specific additions to the basic reaction 1.

First we have included into reaction 1 the opening conformation change of the single-liganded receptor,

$$AR \underset{\alpha_1}{\overset{\beta_1}{\rightleftharpoons}} AR^*.$$

The rate constants derived for this step by Colquhoun and Sakmann (1985) were $\alpha_1 = 6{,}500\ s^{-1}$ and $\beta_1 = 2.3\ s^{-1}$ which corresponds to an observed component of rare and short openings. When this step was added to reaction 1 with otherwise the standard parameters, î was increased by about i/1,000 with 1 μM ACh and by even a lower fraction with 1,000 μM ACh. Also, the changes in t_r were immeasurably small. Thus with the proposed α_1 and β_1 the contribution to the simulated dose-response curves for î and t_r were insignificant. In contrast assuming a higher probability of the AR^* open state (higher β_1/α_1) led to increases in î in the 1–100 μM range of ACh and to much reduced values of t_r for low ACh concentrations. As a result the slope of the dose-response curve for î was reduced to values near 1.

The measured dose-response curves for î in adult muscles had maximal double-logarithmic slopes >2 (Fig. 2). This feature could not be matched by any of the variations in Fig. 4, because reaction 1 contains only two binding steps. To increase the slope, three binding steps seem necessary:

$$\begin{matrix} A & & A & & A & & \\ + & \overset{3k_1}{\rightleftharpoons} & + & \overset{2k_2}{\rightleftharpoons} & + & \overset{k_3}{\rightleftharpoons} & \\ R & \underset{k_{-1}}{} & AR & \underset{2k_{-2}}{} & A_2R & \underset{3k_{-3}}{} & A_3R \end{matrix} \overset{\beta}{\underset{\alpha}{\rightleftharpoons}} A_3R^*. \qquad (2)$$

REACTION 2

Relative to the control (reaction 1) of Fig. 5, addition of a third binding step (reaction 2), keeping the parameters the same, greatly increased the slope of the î curve by depressing the î at low ACh concentrations. The change from reaction 1 to reaction 2 had relatively small effects on t_r.

It has often been discussed that agonist binding might be truly cooperative, i.e., that the first binding of an agonist would improve the rate of binding of the second agonist molecule (Colquhoun and Sakmann, 1985; Sine and Steinbach, 1987; Sine et al., 1990). By lowering k_1 relative to the control value to 10^7 and increasing k_2 to the diffusion limit (see Hille, 1984) of $3 \times 10^8\ M^{-1}\ s^{-1}$, such cooperativity could be achieved (Fig. 6). The results were curious. The î curve was shifted to the right, obviously more affected by the decrease in k_1 than by the increase in k_2. The t_r showed a similar shift, but in addition, a peculiar maximum at 30 μM ACh. Cooperativity thus does not seem to be generally suitable for an approximation of the measured relations.

Approximately optimal fits of the measured dose-response curves

Based on the information regarding the effects of parameter variations in Figs. 4–6, relatively good fits between the experimental results and the model curves could be obtained by selecting appropriate parameters. In Fig. 7, typical dose-response curves measured in adult muscle (*A–D*) or in denervated muscle (*E* and *F*), respectively, are presented as drawn-out curves. The dotted curves represent the respective simulations. The results in adult muscle were fitted well by reaction 1 (*A–B*) with the parameters $k_1 = k_2 = 10^8\ M^{-1}\ s^{-1}$, $k_{-1} = k_2 = 20{,}000\ s^{-1}$, $\alpha = 1{,}100\ s^{-1}$ and $\beta = 50{,}000\ s^{-1}$. These parameters, apart from k_1, are about two times higher than the initial values obtained from Colquhoun and Sakmann (1985). Their parameters were taken from measurements at 11°C, whereas our measurements were at 21°C. The increase in the fitting parameters compared to the initial values is quite likely to reflect the higher temperature.

One possibly essential feature of the î dose-response in Fig. 7*A* which is not fitted by the simulation is the initial slope >2. To increase this slope, the three binding steps in reaction 2 were employed in Fig. 7 *C* and *D*, decreasing $k_{-1} = k_{-2} = k_{-3}$ to $4{,}000\ s^{-1}$ and increasing α. The fit has more than the required initial slope. However, we have observed double-logarithmic slopes of up to 2.7 in other experiments (Franke et al., 1991*a*). As shown above, addition of a significant AR^* component would reduce the slope of the î dose-response. If this AR^* would have had a higher open probability than proposed by Colquhoun and Sakmann (1985), a fit with three reaction steps might produce perfect results.

For the î and t_r measured in denervated muscles, the initial parameters (Colquhoun and Sakmann, 1985) proved to generate the best fits (Fig. 7 *E* and *F*). In denervated muscles the level of t_r at low ACh concentrations tended to vary in different patches, whereas the t_r values at high ACh concentrations were stable (Fig. 3). Also adult muscles showed this variation, but to a smaller extent (Fig. 2). Large variations of the plateau of t_r at low ACh concentrations can best be achieved by changes in α (Fig. 4). These changes of α do not much affect t_r at high ACh concentrations as well as the dose responses of î.

DISCUSSION

The main result of this study is that reaction 1 with the rate constants derived from measurements of single channel kinetics with steady-state application of ACh

(Colquhoun and Sakmann, 1985) predicts very well the responses to pulses of ACh. This agreement provides strong validation of schemes like reaction 1 for the description of receptor-agonist reactions. The congruence of the two approaches is the more remarkable because the steady-state kinetics were obtained from desensitized receptors. Desensitization by high ACh concentrations decreases the rates of channel opening in adult vertebrate muscle to ~î/10,000–î/100,000, and in denervated muscle to ~î/200 (Franke et al., 1991*a*, *b*). Nevertheless, the rate constants of channel opening and closing in the desensitized state seem to agree well with those controlling the initial reaction to an ACh pulse, which occurs before desensitization sets in. Activation and desensitization thus seem to be uncoupled essentially.

If the opening and closing kinetics of channels in the continued presence of the transmitter, and also the rise and the peak of average channel current elicited by a stepwise rise in transmitter concentration yield the same reaction rates, both of these approaches could be used alternatively to estimate such rates. Estimates from the steady-state, open-closed, single-channel kinetics have the advantage that relatively simple equations have been derived which relate average open times, closed times, burst durations and burst intervals, and cluster durations and intervals to the reaction rates $k_{-1} = k_{-2}$, α and β (Colquhoun and Ogden, 1988; Colquhoun and Hawkes, 1981, 1982). $k_{+1} = k_{+2}$ has to be selected freely, guided by the diffusion limit. However, the estimation of average open and closed times becomes very difficult when α and β are large, i.e., when very short openings and closings prevail. Then the evaluation of the recorded currents may be limited by the frequency response of the measuring system. Indeed, it was a major effort and achievement to extract the high α and β rates characteristic for the nicotinic ACh receptors (Colquhoun and Sakmann, 1985). In this, corrections have to be made for unresolved openings or closings, for 'missing events.' These corrections are probable to generate considerable errors.

As discussed in Franke et al. (1991*a*) a difficulty with the assumption of the three binding sites for ACh for one receptor is the accepted localization of the binding sites in the two α subunits of the receptor (Sakmann et al., 1985). A possibility to avoid the three binding sites on one molecule is the assumption that double-liganded receptors form a dimer, and that channels open in this dimer:

$$A + R \rightleftharpoons AR + A \rightleftharpoons A_2R$$

$$2A_2R \rightleftharpoons (A_2R)_2 \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} (A_2R)_2^* \qquad (3)$$

REACTION 3

in which $(A_2R)_2$ is the dimeric receptor and $(A_2R)_2^*$ the open channel. Such dimerization steps, although for AR forms of the receptor, have also been discussed by Colquhoun and Sakmann (1985) and by Colquhoun and Ogden (1988). Simulations of reaction 3 show that it is possible to fit the results on adult muscle channels in this manner, including the slope >2.

The measurement of the average rise time t_r and of the peak current î attained after a step change in agonist concentration is not seriously limited by the frequency resolution of the system. The limits are set not so much by the recording system, but by the rapidity of agonist application. Rise times < 0.5 ms can be reached only for excised patches exposed to shifting jets of solution (Franke et al., 1987; Maconochie and Knight, 1989; Dudel et al., 1990). This restricts the analysis of rapid reaction systems to excised patches. However, slower systems, or rapid systems activated by low agonist concentrations could be probably subjected to t_r and î analysis also in whole-cell configurations, provided the cells can be exposed to jets of solutions.

A disadvantage of t_r and î analysis of rate constants, compared to steady-state channel kinetics, is the present lack of analytical expressions for the relations between the characteristics of the t_r and î dose-response curves and the respective reaction rates. A complicated trial-and-error procedure for curve fitting is necessary, which leads to unambiguous results, but lacks the elegance of simple equations relating the parameters. Mathematical derivation of t_r and î from the set of differential equations describing reaction 1 is quite complex, but may be available soon. For the first step of reaction (1), $A + R \rightleftharpoons AR$, the half-rise time $t_{0.5r}$ of AR can be derived (see Hill, 1909; Mackay, 1977; Colquhoun and Hawkes, 1977):

$$t_{0.5r} = \frac{ln\ 2}{k_1[A] + k_{-1}} \qquad (4)$$

in which [A] is the agonist concentration after the step. Eq. 4 already expresses the features of the t_r dose response, as measured and simulated above. For high $[A]$, $t_{0.5r}$ approaches zero, and for low $[A]$ the asymptote is $(ln2)/k_{-1}$. As seen in Fig. 4, in the complex reaction (1) the low $[A]$ limit of t_r is also approximately reciprocal to k_{-1}. H. Parnas et al. (1989) gave an expression for the rise time which applies to reaction 1. However, their solution was derived for the extremely short ACh pulses generated by quantal release of ACh and the action of cholinesterase. In this case, too, t_r increases when $k_{-1} = k_{-2}$ decreases, but t_r also depends on α and β. Land et al. (1981) derived binding parameters from the duration of the rising phase of spontaneous miniature endplate potentials of lizard muscle. Using reaction 1 they ob-

tained $k_1 = k_2 = 5 \times 10^7\ M^{-1}\ s^{-1}$ and $\alpha + \beta = 25{,}000\ s^{-1}$. Both these rates are about half as large as those obtained by us.

One point in which analysis of single channel kinetics is superior to t_r and î analysis is the definition of the small *AR** component by Colquhoun and Sakmann (1985). This component is too small to influence t_r or î significantly.

The comparison of the rate-constants derived from single-channel kinetics and from the t_r and î dose-responses was not very rigorous; we did not attempt to improve the exactness of the values of the rate constants, but aimed for a validation of the principles. An exact comparison would have to be performed with data obtained in the same preparation and under the same conditions. The preparation of the study of Colquhoun and Sakmann (1985) was frog muscle at 11°C. Pulsed ACh application and measurement of t_r and î will have to be done either in frog or in mouse muscle at 11°C, and in the case of mouse muscle, the single-channel kinetics also need to be determined at 11°C, hoping that at this temperature the short openings and closings can be resolved sufficiently.

Our estimates of the rate constants of ACh-receptor reactions in adult and "denervated" muscle may be compared to estimates of such parameters by Jaramillo and Schuetze (1988) for rat myotubes. They derived the rate constants from the open-closed kinetics of the channels in presence of low ACh concentrations. Because measurements were done at 10°C, all these values are relatively low. For the adult channel type, $\alpha \approx 300\ s^{-1}$, $\beta \approx 23{,}000\ s^{-1}$ and $k_{-2} \approx 11{,}000\ s^{-1}$ were derived. These values are about two times lower than respective ones in Fig. 7 *A* and *B*, and they agree with those (except for a lower *a*) reported by Colquhoun and Sakmann (1985) for frog muscle at 10°C. For the embryoniclike channel, which corresponds to our "denervated" one, Jaramillo and Schuetze (1988) found $\alpha \approx 100\ s^{-1}$, $\beta \approx 6{,}000\ s^{-1}$ and $k_{-2} \approx 3{,}000\ s^{-1}$. The respective fitting values in Fig. 7, *E–F* were $\alpha = 700\ s^{-1}$, $\beta = 30{,}000\ s^{-1}$ and $k_{-2} = 8{,}000\ s^{-1}$. The denervated mouse muscle studied by us thus had 3–7 times higher reaction rates than the rat myotubes which should only partly be due to the higher temperatures. But in both preparations, the tendency of lower reaction rates in "embryonic" as compared to adult nAChRs was the same.

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