

# ACTIVATION OF A NICOTINIC ACETYLCHOLINE RECEPTOR

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**ABSTRACT** We studied activation of the nicotinic acetylcholine (ACh) receptor on cells of a mouse clonal muscle cell line (BC<sub>3</sub>H1). We analyzed single-channel currents through outside-out patches elicited with various concentrations of acetylcholine (ACh), carbamylcholine (Carb) and suberyldicholine (Sub). Our goal is to determine a likely reaction scheme for receptor activation by agonist and to determine values of rate constants for transitions in that scheme. Over a wide range of agonist concentrations the open-time duration histograms are not described by single exponential functions, but are well-described by the sum of two exponentials, a brief-duration and a long-duration component. At high concentration, channel openings occur in groups and these groups contain an excess number of brief openings. We conclude that there are two open states of the ACh receptor with different mean open times and that a single receptor may open to either open state. The concentration dependence of the numbers of brief and long openings indicates that brief openings do not result from the opening of channels of receptors which have only one agonist molecule bound to them. Closed-time duration histograms exhibit a major brief component at low concentrations. We have used the method proposed by Colquhoun and Sakmann (1981) to analyze these brief closings and to extract estimates for the rates of channel opening ( $\beta$ ) and agonist dissociation ( $k_{-2}$ ). We find that this estimate of  $\beta$  does not predict our closed-time histograms at high agonist concentration (ACh: 30–300  $\mu$ M; Carb: 300–1,000  $\mu$ M). We conclude that brief closings at low agonist concentrations do not result solely from transitions between the doubly-liganded open and the doubly-liganded closed states. Instead, we postulate the existence of a second closed-channel state coupled to the open state.

## INTRODUCTION

The function of membrane receptors for neurotransmitters is understood in a general fashion, but a precise description of how receptors are activated by neurotransmitters is lacking. As more information from single-channel current records becomes available, it is becoming clear that the function of the nicotinic acetylcholine (ACh) receptor has complexities that were not appreciated from studies of macroscopic currents. Studies of the dependence of receptor activation on agonist concentration are essential in distinguishing possible schemes for receptor activation and for estimating the rates of transitions between receptor states. Accordingly, we have studied the dose-response relationship for the activation of ACh receptors in outside-out patches of membrane from BC<sub>3</sub>H1 cells.

The ACh receptors produced by these cells have been characterized as nicotinic by biochemical and pharmacological criteria (Boulter and Patrick, 1977; Sine and Taylor, 1979, 1980, 1981). Only one conductance class of channels is present in the cells which we have studied. The kinetic behavior of the channels is qualitatively similar to that of other ACh receptors at similar agonist concentrations (Sakmann et al., 1980; Colquhoun and Sakmann, 1981). Channel open-time histograms are well described by the sums of two exponential components, indicating the

existence of two open states of the receptor. Channel closed-time histograms show a major component of very brief closings at all concentrations, and at high concentrations show multiple (five) apparent exponential components. Our analysis of the concentration dependence of open-time and closed-time histograms has led us to postulate additional states of the ACh receptor. Individual ACh receptors may open to either a brief or long duration open state. There is also a closed state of short duration in rapid equilibrium with the long open state of the receptor that is different from the doubly liganded but closed state (see also Auerbach and Sachs, 1983).

## MATERIALS AND METHODS

Clonal BC<sub>3</sub>H1 cells were maintained as described previously (Sine and Taylor, 1979). For biophysical studies, cells were dissociated with 0.5% Viokase (vol/vol, Gibco Diagnostics, Chagrin Fall, OH) in Dulbecco-modified Eagle's medium. They were plated on glass cover slips and maintained in medium plus 0.5% cadet calf serum (Biocell Lab., Carson, CA) to inhibit cell division and promote differentiation and production of AChR (Patrick et al., 1977). BC<sub>3</sub>H1 cells were chosen because the AChR expressed by these cells has been characterized as a nicotinic ACh receptor biochemically (Boulter and Patrick, 1977; Merlie and Sebbane, 1981), pharmacologically (Sine and Taylor, 1979, 1980, 1981), and in terms of its synthesis and degradation (Patrick et al., 1977). This cell line was derived from an intracranial tumor in a C<sub>3</sub>H mouse (Schubert et al., 1974). Cells were studied at passages 16–45 in different experiments.

We found that high-resistance seals formed more frequently on cells that had been lightly treated with proteolytic enzymes a week before recording. Cells grown on cover slips in 0.5% serum for 5–6 d were rinsed once with serum-free medium, then covered with 0.5% Viokase in serum-free medium. Cells were treated for 5–10 min at room temperature, until the majority of cells were “rounding up” slightly. Medium containing serum was added to the dishes to bring the serum concentration to 2%, then the medium was aspirated and replaced with fresh medium containing 0.5% serum. Cells were studied 14–20 d after being plated on cover slips (8–14 d after Viokase treatment).

Cells were bathed in salt solutions for biophysical studies. Monovalent ion concentrations were usually symmetrical. Extracellular solutions contained agonist, 1.8 mM  $\text{CaCl}_2$ , 1.7 mM  $\text{MgCl}_2$ , and 25 mM HEPES (pH 7.4), whereas intracellular solutions contained 2 mM  $\text{MgCl}_2$ , 1 mM EGTA, and either 10 mM or 25 mM HEPES (pH 7.4). Monovalent ion concentrations used were 140 mM  $\text{Na}^+$  + 5.3 mM  $\text{K}^+$ ; 140 mM  $\text{K}^+$  + 12 mM  $\text{Na}^+$  (cell attached) or 145 mM  $\text{K}^+$ , with  $\text{Cl}^-$  as the major counterion.

For biophysical studies, cover slips were washed several times in salt solution then placed in a chamber over a Peltier temperature-controlling device mounted on the stage of inverted microscope (Diaphot, Nikon Inc., Garden City, NY). Standard patch-clamp methods were used (Hamill et al., 1981), with a homemade patch-clamp circuit based on the design shown in Hamill et al. (1981). The clamp was tuned to have a response time constant of 60  $\mu\text{s}$ . Typical rms base-line noise was  $\sim 0.25$  pA (3,400 Hz) and  $\sim 0.5$  pA (7,800 Hz) with a 20 G $\Omega$  seal.

To study the dose-response relationship with outside-out patches, about four-fifths of the bath volume was aspirated and replaced with an equal volume of the new solution, repeated five times. After the final exchange it took 2–5 min for the bath temperature to equilibrate, after which data were recorded. By this time the channel-opening frequency had reached a new steady-state level. Increasing concentration steps were used in almost all experiments. The experiments ended when the patch broke, so we did not record replicate exposures to a given concentration at the start and end of an experiment.

Data were recorded on FM analog tape (Racal Store 4D, Racal Recorders Inc., Sarasota, FL) and replayed for analysis. Analog records were filtered (Bessel 4-pole or 8-pole filter, Frequency Devices, Inc., Haverhill, Mass.), digitized at 50  $\mu\text{s}$  intervals and stored on magnetic discs using a PDP 11/34 minicomputer (Digital Equipment Corp., Maynard, Mass.). Data reduction was performed by the computer, with operator inspection and approval of each detected transition. Transitions were detected by a threshold-crossing procedure, with the thresholds for both opening and closing transitions set at 50% of the mean open-channel current level. An equipment “dead time” was imposed during data reduction by setting the condition that any transition had to persist for a number of sample points (usually two points). Durations were defined as the number of sample intervals between the first point to pass threshold in one direction and the first subsequent point to return past threshold in the other direction. In these records multiple openings were very rare (<1%). Amplitudes and durations of multiple openings were not analyzed, but the closed times preceding and following them were included in the analysis of closed-time distributions.

Both open-time and closed-time histograms are plotted as the number of durations ending during an interval, not as the total number of durations lasting longer than a given time. Our closed-time histograms are equivalent to the “first-latency histograms” of Dionne and Leibowitz (1982).

All data averages are given as mean  $\pm$  SD;  $N$  = number of observations, unless otherwise stated.

## RESULTS AND DISCUSSION

### General Observations

The most frequently seen channel in patches on  $\text{BC}_3\text{H1}$  cells is the ACh receptor channel. A smaller ( $\sim 10$  pS)

channel is seen in  $\sim 10\%$  of the patches. This channel appears to be  $\text{K}^+$  selective. Its opening does not depend on the presence of agonist but the frequency of seeing it does seem to increase with membrane hyperpolarization. This channel stays active for long periods (up to seconds) and shows frequent “flickering” when open. We did not analyze data from patches in which this channel appeared frequently enough to overlap ACh receptor channel currents.

There is only one amplitude class of ACh receptor channels on  $\text{BC}_3\text{H1}$  cells up to  $\sim 20$  d after plating them in differentiation medium (Fig. 1). We have seen subconductance states (Hamill and Sakmann, 1981) very rarely; 99% or more of all closings lasting longer than 200  $\mu\text{s}$  reach baseline (Fig. 1). With longer time in culture, however, we have seen subconductance states more frequently and a second amplitude class of channels appears. We are currently working to understand the basis of this change in properties. In this paper, however, we shall restrict ourselves to a consideration only of the ACh receptor channels on young cells in culture.

ACh receptor currents are elicited by ACh, carbamylcholine (Carb), suberyldicholine (Sub) and decamethonium. We have not seen any currents in the absence of

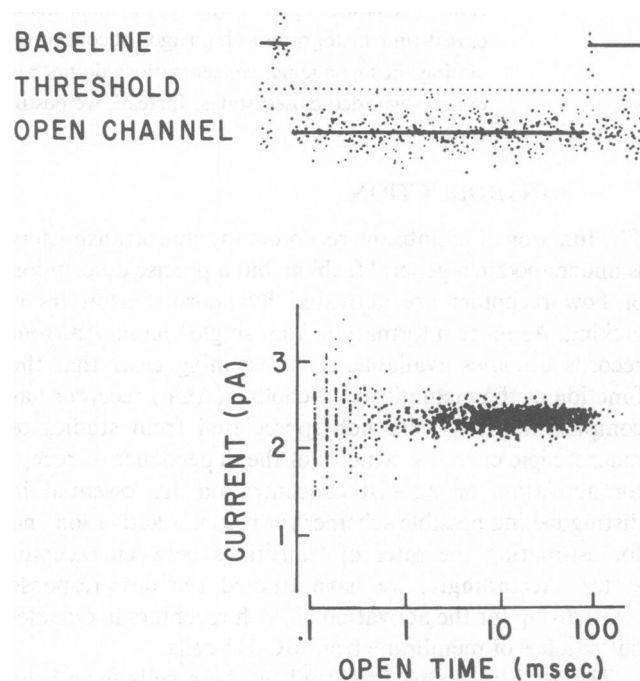


FIGURE 1 Upper panel, a portion of a group of channel openings elicited by 100  $\mu\text{M}$  ACh in an outside-out patch ( $-70$  mV,  $9.8^\circ\text{C}$ ). The record was filtered at 3,400 Hz and digitized at 50- $\mu\text{s}$  intervals. The relationship of the transition threshold (....) to the baseline (upper solid-line segments) and single channel levels (lower solid-line segment) is also shown. The opening shown had a duration of 19.7 ms and an amplitude of  $-2.3$  pA. The closing on the right of the fitted open event lasted for six sample points (300  $\mu\text{s}$ ) and reached baseline. Lower panel, a scatter-plot of the mean current amplitude against the channel open time (plotted logarithmically) for this experiment (1,305 events). There is only one amplitude class of channels present.

agonist which we can ascribe to ACh receptor channels. Treatment of cells with  $\alpha$ -bungarotoxin eliminates ACh receptor currents.

The current-voltage relationship is quite linear in both the inward and outward direction, but there is a bend near the reversal potential in symmetrical  $K^+$  solutions (Fig. 2). The slope depends on temperature (Fig. 2). An Arrhenius plot of conductances over the range 4°–24°C shows no obvious inflection and gives a  $Q_{10}$  of 1.51 ( $E_A$  of 6.85 kcal/mol).

At room temperature the inward conductances in 150 mM  $Cs^+$  or  $K^+$  solutions are similar (in  $K^+$  solutions,  $g = 51.1 \pm 3.0$  pS,  $N = 24$ ) and are greater than the conductance in 140 mM  $Na^+$  + 5.3 mM  $K^+$  ( $g = 33.6 \pm 5.0$  pS,  $N = 16$ ). Conductances are identical with different agonists (for example, at room temperature in  $Na^+$  +  $K^+$  solution,  $g_{(Sub)} = 32.4 \pm 5.0$  pS,  $N = 5$ ;  $g_{(ACh)} = 34.1 \pm 4.5$  pS,  $N = 5$ ;  $g_{(carb)} = 33.2 \pm 2.3$  pS,  $N = 6$ ).

These results indicate that the ACh receptor on BC<sup>3</sup>H1 cells is a typical nicotinic ACh receptor, in agreement with the extensive data available on agonist-induced  $Na^+$  fluxes and agonist and antagonist inhibition of  $\alpha$ -neurotoxin binding (Sine and Taylor, 1979, 1980, 1981).

### High Agonist Concentrations: Channel Block

All agonists we have examined appear to block current flow through the ACh receptor channel (Sine and Steinbach).<sup>1</sup> Channel block occurs at relatively high concentrations of agonist, and complicates the interpretation of dose-response studies. We have characterized channel block to determine the useful concentration ranges of some agonists.

We found that the mean inward current through ACh receptor channels was reduced when high external concentrations of ACh or Carb were present ( $>300$   $\mu$ M ACh or  $>1$  mM Carb) and that the open channel "noise" was increased. The reduction in mean current depends on both membrane potential and agonist concentration. Between 0 and  $-100$  mV the reduction is well-described by a simple ion-blocking model (Woodhull, 1973). The data are consistent with the idea that ACh causes an extremely short-lived block of the ACh receptor channel, with an apparent dissociation constant of  $\sim 50 \times \exp(V \times 0.032 \text{ mV}^{-1})$  mM, for ACh (11°C). Inward currents begin to increase when the membrane potential is more negative than  $\sim -160$  mV, suggesting that agonist molecules can pass through the ACh receptor channel (see Dwyer et al., 1980; Adams et al., 1981). Carb has similar effects, but the apparent  $K_D$  is two- to threefold larger than that for ACh.

Suberyldicholine causes frequent brief interruptions of single channel currents when present at concentrations above 10  $\mu$ M. When Sub concentrations are varied

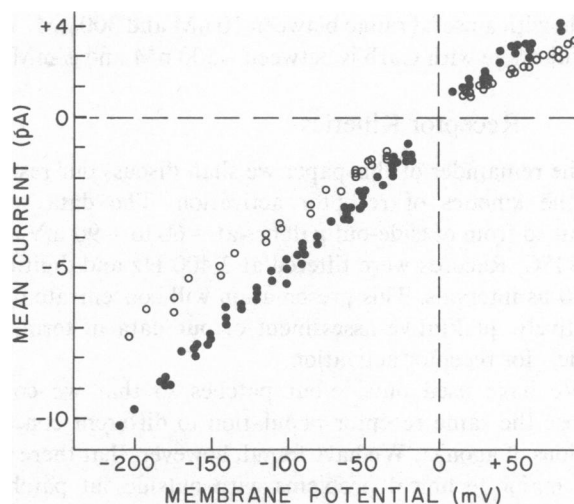


FIGURE 2 Mean single-channel currents are shown plotted against the patch potential, at 20–22°C (●) and 10–12°C (○). Each point is the mean of 50–200 events recorded at a given potential for four patches (10–12°C) or six patches (20–22°C), recorded in symmetrical 150 mM  $K^+$  solutions.

between 10 and 30  $\mu$ M the mean apparent open time decreases with increasing Sub concentration, while at a constant concentration the mean open time decreases with membrane hyperpolarization ( $-50$  to  $-110$  mV). The closed-time duration histograms are more difficult to interpret, since they should depend on agonist concentration in the absence of channel block (Colquhoun and Hawkes, 1981). However, at concentrations higher than 10  $\mu$ M, Sub channel blocking events outnumber state transitions to closed-receptor states. We have found a major component in the closed-time histograms (10–30  $\mu$ M Sub) whose time constant does not depend on Sub concentration but is increased when the membrane is hyperpolarized. The open time and this component of the closed time can be well described in terms of a sequential blocking model, which has been used to describe the blocking action of local anesthetics (Neher and Steinbach, 1978). The forward and backward rates are  $2.2 \times 10^{-6} \exp(-V \times 0.015 \text{ mV}^{-1}) \text{ M}^{-1} \text{ s}^{-1}$  and  $1.14 \times 10^4 \times \exp(V \times 0.017 \text{ mV}^{-1}) \text{ s}^{-1}$  (11°C; 10–30  $\mu$ M Sub;  $-50$  to  $-110$  mV). The apparent  $K_D$  is, then,  $5.2 \times \exp(V \times 0.032 \text{ mV}^{-1})$  mM.

Decamethonium at 30–60  $\mu$ M concentrations produces single-channel currents which are qualitatively similar to those of Sub (10–30  $\mu$ M). Adams and Sakmann (1978) have provided evidence that decamethonium blocks the ACh receptor channel, and Creese and England (1970) have demonstrated that decamethonium passes across the muscle membrane at the endplate region.

These observations define the maximal agonist concentration that can be used without channel block by the agonist becoming the dominant process apparent in single channel current records, and obscuring channel activation processes. The concentration of Sub should be kept under 1  $\mu$ M, which severely limits the usefulness of this agonist. The most suitable agonist of the ones we have tested is

<sup>1</sup>Sine, S. M., and J. H. Steinbach. Manuscript in preparation.

ACh, with a useful range between 10 nM and 300  $\mu$ M. The useful range with Carb is between  $\sim$ 500 nM and 1 mM.

### Receptor Kinetics

In the remainder of this paper we shall discuss our results on the kinetics of receptor activation. The data were obtained from outside-out patches at  $-60$  to  $-90$  mV and  $9^\circ$ – $11^\circ$ C. Records were filtered at 3,400 Hz and digitized at 50  $\mu$ s intervals. This presentation will concentrate on a relatively qualitative assessment of our data in terms of models for receptor activation.

We have used outside-out patches so that we could expose the same receptor population to different concentrations of agonist. We have found, however, that there are two major technical problems with outside-out patches. First, patches are relatively delicate and often do not last for the time necessary for experiments (30–90 min), especially when polarized by more than 70–90 mV. Second, our impression is that patches may suffer a loss of activatable ACh receptors during solution changes. This is evidenced by seeing only a small increase in the rate of channel opening when agonist concentrations are raised in the low concentration range. For this reason, we will not interpret the changes in long closed periods between independent channel openings as concentration is changed.

At low concentration, channel openings occurred either individually, separated by long closed periods, or in small groups with very brief closed periods between them. At high concentrations, channel openings occurred in groups of repeated openings. Groups of openings were clustered, with relatively short closed periods separating groups ( $\sim$ 100 ms). Clusters were separated by long closed periods, often lasting many seconds. These observations are similar to those of Sakmann et al. (1980) and Colquhoun and Sakmann (1981).

The terminology used to describe these phenomena has varied somewhat in the literature. We will use the term “burst” for single or closely-spaced openings at low concentration (Colquhoun and Hawkes, 1981; Colquhoun and Sakmann, 1981); “group” for a series of closely-spaced openings at high concentration; and “cluster” for groups of groups at high concentration.

### Open-Time Durations

The open-time histogram is not a single exponential, but is described well by the sum of two exponentials (Fig. 3), with time constants differing 50- to 100-fold (see also Colquhoun and Sakmann, 1981). Two exponentials are seen at concentrations of ACh between 20 nM and 300  $\mu$ M, Sub between 10 and 200 nM, and Carb between 1  $\mu$ M and 1 mM. The fraction of the total of brief openings (calculated from the fitted curves) was  $0.39 \pm 0.14$  (ACh,  $N = 5$ , 10–500 nM),  $0.26 \pm 0.10$  (Sub,  $N = 7$ , 10–100 nM) and  $0.21 \pm 0.01$  (Carb,  $N = 2$ , 6–10  $\mu$ M). The difference in relative frequencies between Carb and ACh is margin-

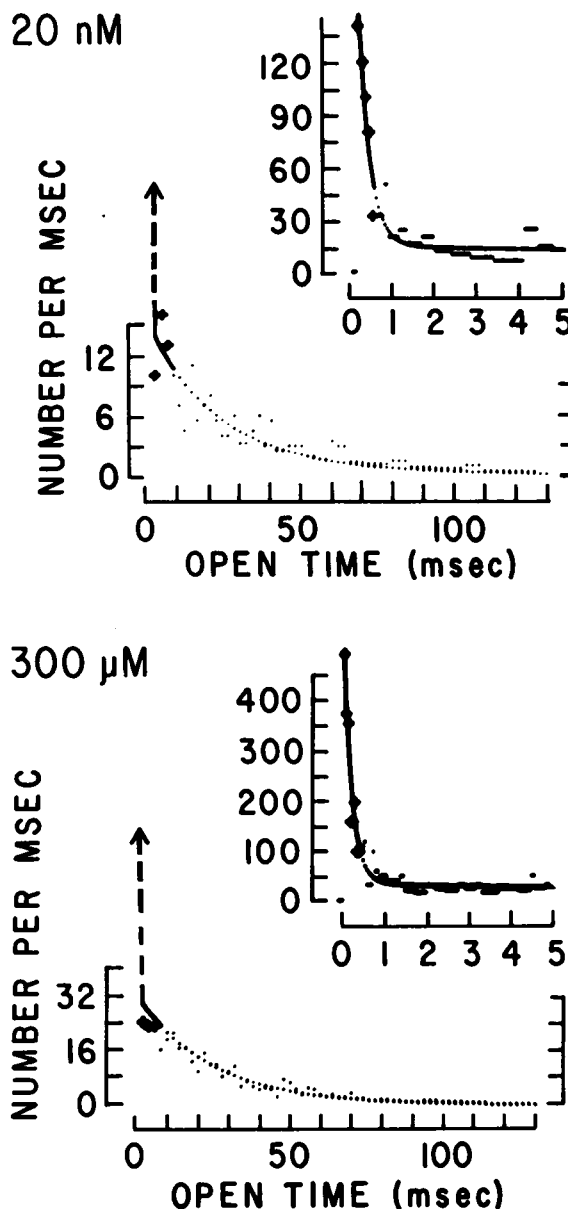


FIGURE 3 Open-time duration histograms from one outside-out patch at low (20 nM, upper panel) and high (300  $\mu$ M, lower panel) concentrations of ACh. Each histogram is shown on two time scales; the insets provide an expanded view of the first 5 ms of the histogram. The first few data points have been plotted as crosses for clarity. Each histogram has been fit by eye with the sum of two exponentials. 20 nM (upper): 432 durations in the histogram,  $k_1$  (decay rate of the brief component) =  $3,920 \text{ s}^{-1}$ ,  $n_1$  (calculated number of events in the brief component) = 81,  $k_2 = 43.2 \text{ s}^{-1}$ ,  $n_2 = 350$ . 300  $\mu$ M (lower): 903 durations in histogram,  $k_1 = 4,993 \text{ s}^{-1}$ ,  $n_1 = 223$ ,  $k_2 = 41.3 \text{ s}^{-1}$ ,  $n_2 = 834$ . Data recorded at  $-70$  mV,  $11^\circ$ C in symmetrical 140 mM  $\text{K}^+$  + 12 mM  $\text{Na}^+$  solutions. Analog signals were filtered at 3,400 Hz, digitized at 50- $\mu$ s intervals and analyzed with a “dead time” of 100  $\mu$ s. The bin size for the lower histograms is nominally 2 ms; for the inset histograms it is nominally 50  $\mu$ s but adjacent bins were summed until at least five entries were present, and the average number per bin plotted. The ordinate scale is shown as the number of durations per ms, to allow direct comparison between the two plots with different bin size. Note that the amplitude of the slower component is much smaller than that of the faster.

ally significant ( $p < 0.1$ ,  $t$ -test). The rate constants fit to the brief openings did not differ between agonists and did not change with concentration (mean  $6,300 \pm 1,100 \text{ s}^{-1}$ ,  $N = 32$ ). The conductance of brief and long openings was identical (e.g., Fig. 1).

The observation that open-time histograms show two exponential components demonstrates that two different open-channel states exist. These states could arise in many ways; for example, two different kinds of receptors with different mean open-times may exist in the membrane. We have found, however, that there is a temporal association between brief and long openings which indicates that a single receptor may produce either class of opening. Basically, at high agonist concentration we find an excess number of brief openings in groups of long openings.

The observation of a temporal association was first made during an analysis designed to indicate whether brief openings at low concentration occurred in groups of openings separated by short closings. Accordingly, we examined the open-time histograms for openings separated by  $< 2 \text{ ms}$  (an interval selected to include brief closings; see below). At low concentration, all of the excess brief openings were found as "isolated" openings, indicating that brief closings did not occur between brief openings. At higher concentrations of ACh and Carb, however, an excess number of brief openings was present in the grouped openings, as well as in the isolated openings.

We further examined the temporal relationship between brief and long openings by analyzing eight records obtained at high agonist concentrations (ACh, 30–300  $\mu\text{M}$ ). The entire record was divided into consecutive 200-ms intervals (trials), an interval chosen to be much longer than the mean open time for long openings. Each trial was then examined to determine whether channel openings had occurred during that interval, and whether openings had resulted in long or brief duration events. The criterion to distinguish long from brief duration openings was the time at which the fitted probability density functions were equal for the two distributions (see Jackson et al., 1983). We then reasoned as follows. If long and brief duration openings arise from independent processes, the conditional probability of seeing a brief opening in a trial that showed one (or more) long openings should be equal to the overall probability of brief openings across the record. This prediction is clearly violated.

For example, the experiment whose data are illustrated in Fig. 3 gave the following results (300  $\mu\text{M}$  ACh). 115 brief openings were classified in 3,996 trials, giving an estimated probability of 0.0288 per trial. 238 trials contained one or more long openings. Of these 238 trials, 190 had no brief openings, 38 one, 9 two, and 1 three brief openings. Using the criterion for brief and long openings, 19 long openings of a total of 807 would have been erroneously classified as brief. Therefore, at least 29 of these 238 trials with long openings also contained brief openings. The prediction from the Poisson equation,

assuming that brief and long openings are independent, is that only 6.7 of these trials should have had one brief opening, whereas 231 should have had no brief openings and 0.3 should have had more than one. A  $\chi^2$ -test is not reliable, because of the small numbers of predicted trials containing two or more brief openings. However, even the first two terms (zero and one brief opening) give a  $\chi^2$  value of 71, which with one degree of freedom is significant at  $< 0.0005$ . Similarly, the normal approximation to the binomial distribution gives the probability of 29 successes in 238 trials as  $< 0.0001$  in this experiment. Comparable observations were made in all eight experiments, demonstrating that long and brief openings are temporally associated at high agonist concentrations.

Long openings occur nonrandomly through the record at high concentrations, as evidenced by the appearance of groups and clusters. Grouping has been suggested to result from transitions of a single ACh receptor between desensitized and activatable states (Sakmann et al., 1980). The temporal association which we observe, then, indicates that a single ACh receptor may open with either a long or a brief mean open time.

It has been suggested (Colquhoun and Sakmann, 1981) that a brief opening of the channel might result when only one agonist molecule is bound to the ACh receptor (see Fig. 4A). If this were the case, brief openings should become relatively less abundant as the agonist concentration is raised. This does not appear to be the case. A simple prediction is made from the activation scheme shown in Fig. 4A. As the agonist concentration is raised, the equilibrium probability of being in state  $A_2R^*$  should increase relative to that of being in  $AR^*$ , given that a receptor is activatable. For this scheme,  $P(A_2R^*)/P(AR^*) = K_2O_2[A]/O_1$  where  $K_2$  is the association constant for the second binding step,  $[A]$  is the agonist concentration, and  $O_1$  and  $O_2$  are opening equilibrium constants. We estimated  $P(A_2R^*)$  and  $P(AR^*)$  from the total times spent in brief or long openings during a record (calculated from the fitted exponentials, to correct for limited time resolution). The ratio of these probabilities should not be distorted by the presence of desensitization, since the total time spent in an activatable state is cancelled in the calculation. Our data clearly do not meet this prediction: brief openings are far too prevalent at high concentrations (Fig. 5).

At present we favor the idea that brief openings arise from a separate state of the receptor, in relatively rapid equilibrium with the "normal" activatable state which gives rise to long openings. This hypothesis could explain some features of the closed time histograms at high agonist concentrations (see below).

We have indicated this additional brief open state ( $A_nQ^*$ ) in the activation scheme shown in Fig. 4B. We do not know how it is coupled to the R state, as indicated by the open brackets. It is also possible that brief openings could arise from several states of the receptor. For example, at low agonist concentration the brief openings

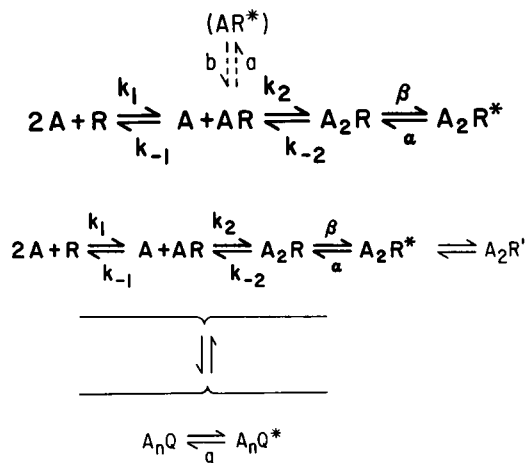


FIGURE 4 Two hypothetical activation schemes for the ACh receptor ( $R$ ) by agonist ( $A$ ). A four-state linear scheme is shown in bold letters in both the *upper* and *lower* panels: two agonist molecules bind to a receptor with a closed channel, then the channel opens ( $A_2R^*$ ). Agonist-induced ion flux studies have shown that open channels in these cells are associated with receptors which have two bound agonist molecules (Sine and Taylor, 1979, 1980). The possibility that receptor channels may open when only one agonist molecule is bound is shown by the dashed arrows and ( $AR^*$ ) in the *upper* panel (Dionne et al., 1978). Association constants for agonist are  $K_1 = k_1/k_{-1}$ ,  $K_2 = k_2/k_{-2}$  and the forward isomerization constants are  $O_1 = b/a$  and  $O_2 = \beta/\alpha$ . The *lower* panel illustrates our current picture of ACh-receptor function. Our data indicate that brief openings do not result from  $AR/AR^*$  transitions. Instead, we postulate another closed state of the receptor,  $A_nQ$ . The  $Q$  and  $R$  states are interconvertible (see text), but the relationship between  $Q$  and  $R$  states and the interconversion rates are not known. The closing rate for  $A_nQ^*$  ( $q$ ) is larger than that for  $A_2R^*$  ( $\alpha$ ). Also shown is an additional closed state,  $A_2R'$ , shown coupled to  $A_2R^*$ . The existence of this state is postulated as a result of our analysis of closed-time histograms. This state does not result from agonist block of the channel (see text).

could reflect the existence of  $AR^*$  whereas at high concentration they could reflect the opening of channels associated with a desensitized state of the receptor. However, because brief openings appear identical at low and high agonist concentrations, we have adopted the hypothesis that all brief openings result from a single state of the receptor.

An association between brief and long openings at high agonist concentrations has been found in studies of purified ACh receptors reconstituted in lipid bilayer membranes (Montal et al., 1983). Jackson et al. (1983), however, have found that the open times of channels separated by brief closings are correlated at lower agonist concentrations—pairs of long or brief openings occur preferentially. Our data do not conflict with this observation, although we find that at low concentrations brief openings occur as isolated events while closely-spaced pairs appear to be of long openings only (see above). The association of brief and long openings is clear only in the groups of openings at high agonist concentrations. Cull-Candy and Parker (1982) have concluded that single glutamate receptors may open to either brief or long duration open states.

A possible artifactual source of the excess brief openings

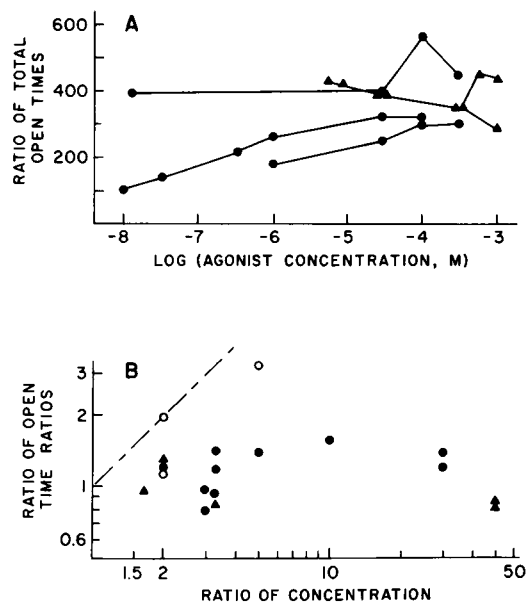


FIGURE 5 *Upper panel*, a plot of the ratio of the total open time spent in long openings to that spent in brief openings during a record, against the agonist concentration applied to outside-out patches (plotted on a logarithmic scale). Data obtained from the same outside-out patches are connected (ACh:  $\bullet$ , Carb:  $\blacktriangle$ ). There is little change in the ratio of open times with change in concentration. *Lower panel*, a plot of reduced data. A series of agonist concentrations was applied to an outside-out patch and the ratio of the total open times was computed for records at each concentration (see *upper panel*). This plot shows the ratio of these computed ratios at two neighboring concentrations, plotted against the ratio of the two concentrations. For example, if three concentrations were applied (10 nM, 50 nM and 500 nM), two points are plotted: ratio(50 nM)/ratio(10 nM) vs. 50 nM/10 nM and ratio(500 nM)/ratio(50 nM) vs. 500 nM/50 nM. The dashed line (---) indicates the relationship predicted (see text). Two points fall close to this line; all of the rest show little change in the open-time ratio with a change in agonist concentration (Sub,  $\circ$ ; ACh,  $\bullet$ ; Carb,  $\blacktriangle$ ).

must be considered: they could result from transient patch breakdown. We have not ruled out this hypothesis, but offer the following observations. The conductance during brief openings is the same as that during long openings (e.g., Fig. 1). The relative frequencies of brief and long openings appear to be related to the agonist present (see above). Finally, the frequency of artifacts would have to be increased by current flow through ACh receptor channels during high concentration bursts (see above). However, overlap between brief and long events during high concentration bursts is not frequent enough for the processes to be likely to be independent. (For example, no multiple openings were detected in the 300  $\mu$ M ACh experiment shown in Fig. 3 and analyzed above, although the open duty cycle during bursts was close to 0.9.) For these reasons we feel that most of the excess brief openings are not artifactual in origin.

### Closed-Time Durations

The distribution of closed times between successive channel openings contains information on the rate of receptor

activation (see Colquhoun and Hawkes, 1981). For example, at low agonist concentration it would be expected that some receptor channels would close and then reopen immediately, before bound agonist molecules had dissociated (see Colquhoun and Sakmann, 1981; Dionne and

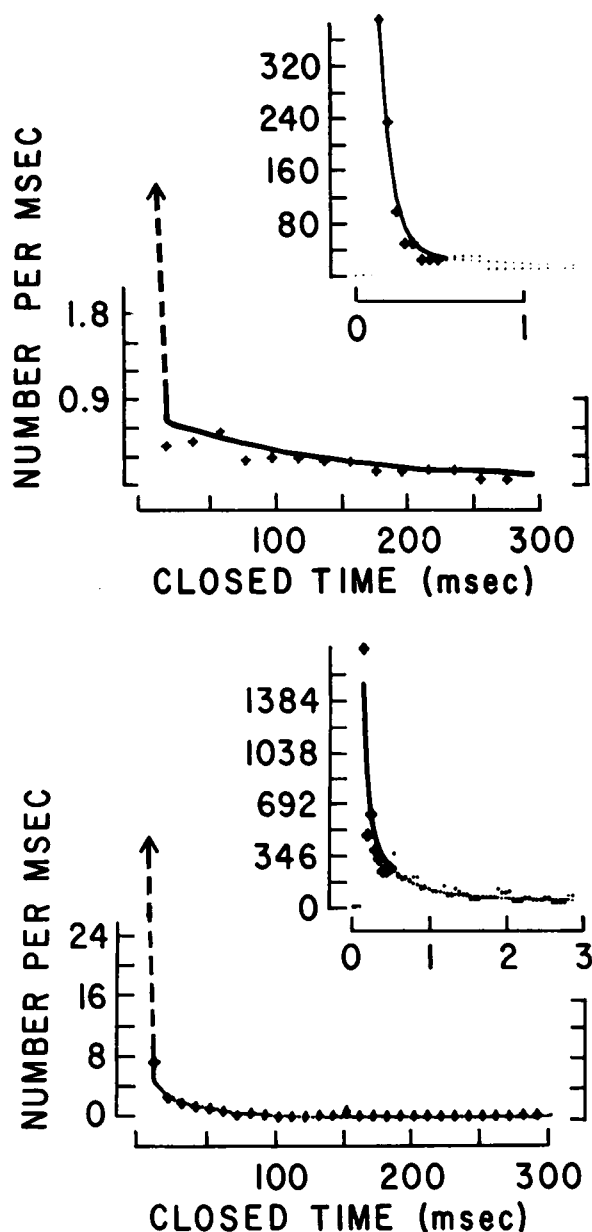


FIGURE 6 Closed-time histograms for the same outside-out patch whose open-time histograms are shown in Fig. 3. The very long-duration closed periods ( $>300$  ms) are not shown in these plots. A series of exponential functions were peeled from the closed-time histograms by eye. For the 20-nM data (431 durations in histogram) four exponentials were peeled, with rate constants and total numbers of durations of  $0.34$   $s^{-1}$ , 307;  $9.30$   $s^{-1}$ , 78;  $720$   $s^{-1}$ , 54; and  $13,720$   $s^{-1}$ , 385. For the  $300$   $\mu$ M data (902 durations in histogram) five exponentials were peeled, with rates and numbers of:  $0.82$   $s^{-1}$ , 90;  $28.7$   $s^{-1}$ , 221;  $337$   $s^{-1}$ , 267;  $2,693$   $s^{-1}$ , 290; and  $18,200$   $s^{-1}$ , 1,180. The bin size for both inset plots is  $50$   $\mu$ s. The bin sizes for the long duration histograms are  $20$  ms ( $20$  nM, upper) and  $10$  ms ( $300$   $\mu$ M, lower). For clarity, the data points are all plotted as crosses in the long-duration histograms.

Leibowitz, 1982). The number and duration of short closed periods should reflect the rates of channel opening and agonist dissociation. At high agonist concentration, where the probability is large that a receptor has agonist molecules bound, the channel would close and reopen repeatedly. In the simplest case, the duration of short closed periods should reflect only the rate of channel opening at high agonist concentrations. We have analyzed closed-time histograms over a range of agonist concentrations with the goal of determining the rates of agonist binding and dissociation and of channel opening.

At low agonist concentrations ( $<200$  nM Sub,  $<500$  nM ACh,  $<10$   $\mu$ M Carb) the closed-time histogram shows a major component of very brief closings (Fig. 6; see also Colquhoun and Sakmann, 1981). A second component, with a time constant of  $1$ – $2$  ms, is often seen, containing  $\sim 1/10$  as many intervals as the rapid component (calculated from the fitted exponentials). Colquhoun and Sakmann (1981) have reported seeing such a component, but with less prevalence. In general one other component is seen with a time constant of seconds, probably reflecting activation of independent ACh receptors. On occasion we have seen a component with a time constant of hundreds of ms (Fig. 5), whose origin is obscure.

The most rapid component of the closed-time histogram (called *nachschlag* here for brevity) is seen with all agonists, although the mean number per burst and the time constant differ between agonists (Table I). Nachschläge do not result from agonist block of the ion channel (see above), and the agonist dependence of their parameters suggests that they result from some process associated with

TABLE ONE  
MEASURED NACHSCHLAG PARAMETERS AND  
CALCULATED RATE CONSTANTS AT LOW  
AGONIST CONCENTRATIONS

	Rate ( $k$ )	"Burst" Length	Number per burst	$\beta$	$k_{-2}$	$\alpha$
	$s^{-1}$	ms	$n$	$s^{-1}$	$s^{-1}$	$s^{-1}$
Carb (2)	16530 (1438)	22.2 (0.3)	0.39 (0.13)	4635 (1492)	11894 (55)	63.5 (13.4)
ACh (5)	14713 (1438)	26.5 (3.7)	1.22 (0.28)	7749 (1307)	6964 (305)	77.9 (5.7)
Sub (8)	13323 (1663)	36.2 (10.1)	1.02 (0.36)	6570 (1511)	6753 (1380)	54.6 (13.8)

Measured parameters are shown in the center three columns, for the agonists (number of experiments) shown at the left. Numbers are mean (SD) of the rate fitted to the nachschlag component ( $k$ ), the average "burst" length (Colquhoun and Sakmann, 1981) and the calculated mean number of nachschläge per burst ( $n$ ). The columns on the right give estimates for the channel opening rate ( $\beta$ ), the agonist dissociation rate ( $k_{-2}$ ) and the channel-closing rate ( $\alpha$ ) calculated from the measured parameters ( $k = \beta + k_{-2}$  and  $n = \beta/k_{-2}$  at low agonist concentration, see Colquhoun and Sakmann, 1981). Neither the measured nor calculated parameters showed changes with agonist concentration in the range examined here (Sub  $10$  nM –  $100$  nM, ACh  $10$  nM –  $500$  nM, Carb  $6$  –  $10$   $\mu$ M).

receptor activation. Colquhoun and Sakmann (1981) have suggested that nachschläge reflect transitions between  $A_2R$  and  $A_2R^*$  in the four-state activation scheme shown in Fig. 4. They have presented a method to extract estimates of the rate constants for channel opening ( $\beta$ ) and agonist dissociation ( $k_{-2}$ ) by analysis of nachschläge (see also Colquhoun and Hawkes, 1981). When this approach is applied to our data the estimates shown in Table I are obtained. Our estimates for  $k_{-2}$  and  $\beta$  with Sub are similar to those obtained by Colquhoun and Sakmann (1981). Our estimate for  $\beta$  with ACh is considerably larger than that made by Dionne and Leibowitz (1982).

Our estimates for  $\beta$  are large for all three agonists, particularly in comparison to the channel-closing rate ( $\alpha$ ). ACh and Sub are considered to be effective agonists in that the receptor ion channel is likely to be open when two agonist molecules are bound to a receptor ( $\beta > \alpha$ ). Previous experiments have suggested, however, that Carb is a less effective agonist and that, for Carb,  $\beta$  and  $\alpha$  are approximately equal (see Adams, 1981).

At high agonist concentration agonist binding will saturate. For the linear activation scheme shown in Fig. 4 A (heavy lettering), the closed-time histogram will show a major fast component with a decay rate approaching  $\beta$  (Colquhoun and Hawkes, 1981). Desensitization processes will produce two slower components reflecting intervals between "groups" and "clusters" of openings (Sakmann et al., 1980). Accordingly, we examined closed-time histograms obtained over a range of agonist concentrations to estimate  $\beta$  both from nachschläge at low concentration and independently from the major fast component at high agonist concentration. ACh was applied at concentrations from 20 nM to 300  $\mu$ M, and Carb at 6–1,000  $\mu$ M. The high concentrations are in excess of the estimated dissociation constants for receptor activation by these agonists, especially in the case of ACh (for ACh,  $K_D$  estimates are  $<40 \mu$ M, for Carb  $< 300 \mu$ M; see Sine and Taylor, 1980; Steinbach, 1980; Karlin, 1980; Adams, 1981).

Qualitatively, we find that our closed-time histograms at high agonist concentrations contain many (five) apparent exponential components. The slowest component, with a time constant of seconds, we associate with intercluster intervals, while the next slowest (30–100 ms) we associate with intergroup intervals (Sakmann et al., 1980), as these phenomena are clearly apparent in our records. However, there remain three more apparent components. A major component is very similar to the nachschläge seen at low concentration; the decay rate of this component shows no consistent change over the entire concentration range studied. Similarly, the fraction of the total closed intervals calculated to belong to this component does not change consistently with concentration. The remaining components have decay rates which differ by factors of 6–10 lying in the ranges of 1,000–3,000  $s^{-1}$  and 100–300  $s^{-1}$ . In particular, we do not see the appearance of a major component with the decay rate predicted by our estimates

of  $\beta$  obtained at low concentration (ACh: 7,500  $s^{-1}$ ; Carb: 4,500  $s^{-1}$ ).

An explanation of our failure to observe the predicted component with a decay rate of  $\beta$  is that even the highest agonist concentrations are still in the low concentration range. We used the equations of Colquhoun and Hawkes (1981) to calculate a predicted distribution of closed times within "apparent bursts" for a linear model, using our estimates of  $\beta$  and  $k_{-2}$  and altering the assumed  $K_D$ . These calculations indicated that a major component with a decay rate approaching  $\beta$  should have been apparent in our closed-time histograms at high concentrations if the  $K_D$  for ACh were  $< 200 \mu$ M or for Carb  $< 600 \mu$ M. Other estimates of the  $K_D$ 's are less than these values (see above).

A second possible explanation is that a component with a decay rate of  $\beta$  is not seen because of the presence of transitions between long- and brief-duration opening states within groups of openings. If doubly liganded receptors ( $A_2R$ ,  $A_2R^*$ ) undergo transitions to other states ( $A_2Q$ ,  $A_2Q^*$ ) then multiple components would appear in the closed-time histograms whose amplitudes and decay rates would depend on the transition rate constants. If the rates from R-states to Q-states were appreciable relative to  $\beta$  and  $\alpha$ , then a component with a rate close to  $\beta$  would not be seen. As we do not know how long- and brief-opening states are connected, we cannot assess the importance of this explanation.

At present, we feel that it is likely that our estimates of  $\beta$  from nachschläge are too large. This conclusion is based on three qualitative impressions. First, our estimates for  $\beta$  are large for all agonists. Second, we cannot account for the presence of an intermediate component in the closed-time histogram at low concentrations. Third, although the closed time histograms at high agonist concentrations are undoubtedly difficult to interpret, the continued presence of a fast (nachschlag) component and absence of a component with the predicted rate suggest that nachschläge may arise from additional processes. We think that it is likely that nachschläge are generated, at least in part, by a transition from  $A_2R^*$  to a low conductance state other than  $A_2R$  (shown as state  $A_2R'$  in Fig. 4 B). Auerbach and Sachs (1983) have demonstrated this to be the case for at least some brief closings of ACh receptors on chick muscle cells in culture. If nachschläge at low concentration do not result solely from transitions between  $A_2R$  and  $A_2R^*$ , then  $\beta$  will be overestimated (see Colquhoun and Sakmann, 1981). At present, we cannot determine what proportion of the nachschläge reflects  $A_2R/A_2R^*$  transitions. Similarly, we cannot associate the intermediate component (rate  $\sim 1,000 s^{-1}$ ) in our closed time histograms at low concentration with particular state transitions.

In summary, we set out to associate kinetic parameters measured from open-time and closed-time histograms with particular steps in receptor activation. To make these associations we varied the agonist and the agonist concen-



tration applied to outside-out patches. Our data do not conform to the predictions of a simple scheme for ACh receptor activation.

The open-time histograms show that the receptor has two open states differing in mean channel open time. A single receptor may open to either state. The dependence of the numbers of brief and long openings on agonist concentration make it unlikely that all of the brief openings occur from receptors with only one bound agonist molecule ( $AR \rightleftharpoons AR^*$  in Fig. 4). We have postulated additional closed and open states of the receptor ( $A_nQ \rightleftharpoons A_nQ^*$  in Fig. 4), for which the closing rate ( $q$ ) is much larger than that for  $A_2R^*$  ( $\alpha$ ). It is not known how R and Q states are interconnected.

Our data on closed times at different agonist concentrations indicate the existence of a second low-conductance state (state  $A_2R'$  in Fig. 4) coupled to the open state of the receptor, which produces at least some of the brief closings seen at all agonist concentrations.

Our conclusions apply specifically to the nicotinic AChR on BC<sub>3</sub>H1 cells, but we feel that they may be generalized to other ACh receptors. Our observations are qualitatively identical to those made on a number of nonjunctional nicotinic AChR (e.g., Sakmann et al., 1980; Colquhoun and Sakmann, 1981; Jackson et al., 1983; Auerbach and Sachs, 1983), and on purified ACh receptors from *Torpedo* (Montal et al., 1983). Indeed, the absence of conductance sublevels in the cells we have studied makes the function of these receptors relatively simple. The AChR at snake neuromuscular junctions may well have simpler activation kinetics (Dionne and Leibowitz, 1982; Leibowitz and Dionne, 1983). To date, however, junctional receptor function has only been studied at room temperature, at which rapid processes may be obscured.

We chose to study AChR function on clonal cells because of the homogeneity of the cell population and the relative ease with which clonal cell lines may be experimentally manipulated. Elucidation of the relationship between the function and structure of the acetylcholine receptor requires defined cell populations with receptors of known function in which receptors may be studied biochemically. The final goal, an analysis of the mechanisms by which the cell controls the expression of functional classes of receptors requires further that the history and condition of the cells be known and controlled. With recent progress in techniques for studying receptor function and biochemistry this goal is achievable in the relatively near future.

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## DISCUSSION

*Session Chairman: Harold Lecar Scribes: Malcolm Hunter and John W. Hanrahan*

KOSOWER: Short and long open states might arise, whatever the structural model, from the nature of the ACh receptor. It could be  $\alpha$ ,  $\beta$ ;  $\alpha$ ,  $\gamma$ ,  $\delta$ ; or  $\beta$  and  $\gamma$  might be switched — see my paper at this meeting. This might apply to any structural model. If you put the ACh in one way, a particular type of opening occurs, whereas if you put it in another way, you may see a similar, but not exactly the same, type of opening. If the binding site is in the channel, this might account for the two different types of openings.

PATLAK: There is a certain amount of confusion about names for the various groupings of openings and closings. Steinbach has a very sensible set of terminologies in this paper that might be universally adopted. At low concentrations of agonist, one usually sees openings that are interrupted by brief closures such as the “Nachschläge” phenomenon, i.e., openings associated with one another. They have been referred to in the literature as “bursts.” The original desensitization phenomena, referred to as “paroxysms” by Montal and “bursts” by Sakmann et al. (Sakmann, E., J. Patlak, and E. Neher. 1980. *Nature (Lond.)* 286:71–73) is a much longer grouping of channel openings. Joe Henry Steinbach has called these “groups.” There is an even longer process, an association of these groups themselves; Steinbach has followed the Sakmann et al. terminology by calling these “clusters.”

My question has to do with the variability of these channels with age of the cultured cells. Do you think the channels that are present on day 10 are still present on day 18, and that these are subjected to some time-dependent change? Or are the cells synthesizing new but different channels?

STEINBACH: The half-life of these receptors is ~12 h, so it is extremely unlikely that the channels present on day 10 are still present on day 18.

DANI: There were often five exponentials in the closed-time distributions that you showed. Is it possible that some of these components are arising because channels are being lost in a way that is different from desensitization, and might be referred to as “run-down” in other preparations. I notice that I lose channels, and it isn't some artifact of the patch-clamp technique such as sealed vesicles.

STEINBACH: Particularly at high concentrations, if you plot the opening frequency over intervals through the record, the regression line is flat, the slope is not significantly different from zero. With regard to long term stationarity, I don't know how to test exactly how stationary these records are because they are too short to detect very slow changes.

HORN: The complexity of the kinetics could mean that each channel has very complex kinetics, or that there is a heterogeneous population of channels having the same conductance. Is it possible to detect channel heterogeneity? Do you have any evidence that this is a homogeneous population other than their having the same conductance?

STEINBACH: First, we think that although brief and long duration open states are present, they both can be produced by a single receptor. The close temporal relationship of brief and long events at high agonist concentration indicates that it is very likely that either the same receptor channel produces both the brief and long openings or, if two different channels are producing them, there must be strong negative cooperativity to prevent multiple openings with such high duty. For that reason, I think that although the open states are heterogeneous, they arise from a single population of ACh receptors.

We analyzed the mean open time of long duration events in groups that contain brief openings and those that do not contain them, to test the idea that there are two types of channels, one which can make a brief opening and one which cannot. There is no difference there. We counted up the number of groups of openings at different concentrations which had one long opening, two long openings separated by a brief closing and so forth. This tests whether the probability of reopening during a short interval after a closing is constant regardless of the number in the train. In other words, is there one type of channel in terms of the grouping behavior? Plotting the number of groups with  $N$  events vs.  $N$  on a semi-log plot, the data fall on a straight line, consistent with the idea that grouping behavior is homogenous.

I think that the grouping and the open time behavior are homogeneous. The single channel conductance also shows no dependence on duration. In the older cells, the channels are heterogeneous.

HORN: What about patch-to-patch variation?

STEINBACH: The measured parameters are certainly within twofold or so, a standard error of  $\pm$  half the mean would be the range. This is similar to the variability that I measured in voltage clamp experiments on neuromuscular junctions.

KOLB: How do you prove in single channel recording that the kinetic process is in equilibrium so that you can apply chemical reaction kinetics?

STEINBACH: I do some tests for stationarity by comparing the event frequencies and mean open times at the beginning and end of a record. There are no significant changes over the length of a 10-min record. On a shorter time scale I do not see characteristic asymmetries; for example, the distributions of durations of closed periods preceding a long opening and following a long opening appear to be time-symmetrical.

KOLB: Regarding the second open state, this might be the case where one ligand binds to the receptor, but do you have any experimental evidence concerning the modulation of the channel from the cytoplasmic side? For example, one of the open states might be generated by the phosphorylation or dephosphorylation of membrane components related to the ACh-activated channel, similar to that found for the Ca-channel in cardiac muscle cells.

STEINBACH: We don't have any evidence regarding that.

KOLB: I want to point out that when one deals with several open states, one should look at modulations of the channel from both the inside and outside.

STEINBACH: We do have indirect evidence that this is not responsible for the two open states because the proportion of brief and long openings is similar in cell attached, inside-out or outside-out patches. We have done a few experiments changing the Ca concentration on the cytoplasmic side and it doesn't seem to affect the relative proportions of brief and long openings. We haven't looked at phosphorylation. We are very interested in the possibility of modification or modulation of receptor function from the cytoplasm.

BENNETT: Why can't the Nachschläge phenomenon be due to a closed state on the other side of the open state (i.e., to the right in the kinetic scheme)? Can miniature post-synaptic currents answer this question? Could one apply what amounts to a  $\delta$  function of transmitter, which I suppose one still can't do with a patch or by iontophoresis? Post-synaptic currents at some snake neuromuscular junctions and at the hatchet fish giant synapse show a rapid decay followed by a slow decay. These data can be explained by assuming that the channel goes from a bound-open state to a bound-closed state; the sequence must be reversed before the transmitter can dissociate. Is there still ambiguity regarding whether the Nachschläge are due to a closed state to the left or right of the open state?

STEINBACH: The lack of concentration-dependence of the frequency and time constant of the Nachschläge in our data indicate that the brief closings do not result exclusively from closures back to the left. At least some of them must result from closed states to the right of the open state, and this closed state is not due to block of the channel by agonist.

DILGER: Working on the same cell line, we have developed a technique of adding agonist within  $\sim 100$  ms to an outside-out patch. We find that several hundred channels are opened in a patch and the number of excitable channels in the patch does not decrease appreciably after 20 min.

LABARCA: You find that the frequencies of the two open states don't change with agonist concentration and the lifetimes also do not seem to change. We have made that observation with purified receptors with several agonists and different concentrations.

SCHNEIDER: I wish to make a general comment about proteolytic enzymes. These may not be completely inactivated by quenching in 2% calf serum. Looking at requirements for synthesis of neural antigens in retinal cultures we have frequently seen lag phases lasting for several days before appearance of the antigens after light trypsinization. We are able to eliminate these lag phases completely by quenching with phenyl methyl sulphonyl fluoride. This lag phase is probably due to adsorption of trypsin

onto the cells; the trypsin can remain there for several days despite exhaustive washing. Also, since you have used digestion to enhance the probability of achieving high resistance seals, are the properties altered (with respect to those observed in untreated cells) by this process?

STEINBACH: We always wait at least four days (six to seven half-lives of the receptor) after enzyme treatment, so we believe the receptors we are looking at were not treated. Based on the data that we have, there is no difference between untreated and treated cells.

SACHS: A general comment: If one has agonist in the pipette, one is looking at the steady-state, and desensitization may have already occurred. This would select for channels that do not desensitize, and these might be different from those that determine the macroscopic properties. There is a discrepancy between the number of channels determined by toxin binding ( $200\text{--}1,000/\mu^2$ ) vs. those calculated from binomial analysis in patches (3–6 channels).

STEINBACH: We do not see changes in properties of the open receptor channel over the concentration range studied here, so the selection seems to be concentration independent. As Dr. Dilger said, he has found better agreement in binding site and receptor numbers using rapid agonist applications.

MOCZYDLOWSKI: You have provided an estimate of  $k_{-2}$ , the agonist dissociation rate. How do these values compare with those obtained from binding studies? Could you use an agonist with a long lived residence time as a tool for identifying a certain fluctuation with agonist residency?

STEINBACH: We hope to do this using curariform antagonists as agonists. You would predict from their  $K_D$  that residency times would be very long. I do not know of reliable biochemical estimates of the off rate constants for strong agonists.

ROUX: Could you explain how you know that the behavior does not arise from independent processes?

STEINBACH: We demonstrate temporal relationships that could not occur through random association of independent receptor activations. A trials analysis showed that brief openings occurred far too frequently in trials that had long openings given the overall probability of a brief opening occurring in any trial, independent of whether there was a long opening in it. An alternative method is to look for time correlations, to demonstrate temporal relationships between the end of one event and the beginning of others. Looking for such temporal relationships is one of the advantages of the patch-clamp technique.