

Direct measurement of the concentration- and time-dependent open probability of the nicotinic acetylcholine receptor channel

James P. Dilger and Roger S. Brett

Departments of Anesthesiology and Physiology and Biophysics, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, New York 11794-8480

ABSTRACT Using the outside-out patch clamp recording technique together with a rapid solution exchange system, we measured ionic currents through nicotinic acetylcholine (ACh) receptor channels from BC3H-1 cells in response to rapid applications of 0.3–1,000 μ M ACh. We used nonstationary fluctuation analysis of ensembles of responses to deduce the number of channels in the patch, the maximum open channel probability as a function

of ACh concentration and the time course of a fast desensitization process. We found that:

(a) Excised patches from BC3H-1 cells typically contain between 50 and 150 functional ACh receptor ion channels.

(b) The open channel probability is proportional to $[ACh]^{1.95}$ at low concentrations of ACh, is half-maximal at 20 μ M ACh and saturates above 100 μ M ACh.

(c) ACh is a very efficacious agonist; 100 μ M ACh opens at least 90% of the available channels. This estimate of efficacy is model-independent.

(d) The rate of decay of the agonist-induced current is concentration-dependent. In the presence of 100 μ M ACh the current decays with a time constant of 50–100 ms. It decays more slowly in the presence of lower concentrations of agonist but is relatively insensitive to voltage.

INTRODUCTION

The efficacy of a drug or neurotransmitter, defined in the classical sense by Stephenson (1956), is a quantitative measure of the ability of a substance, once bound to its receptor, to cause an effect. In the case of the nicotinic acetylcholine (ACh) receptor-ion channel complex, the immediate effect of agonist binding is the opening of the ion channel.

Although the binding of agonist to the nicotinic receptor causes an increase in the conductance of the muscle endplate, prolonged exposure to agonist results in a reversible time-dependent decrease in conductance—desensitization (Katz and Thesleff, 1957). This phenomenon has complicated the measurement of the efficacy of agonists at the ACh receptor channel. A variety of experimental techniques have been tried to circumvent the problem of desensitization. These include electrophysiological recording during fast bath perfusion of agonists (Adams and Feltz, 1980; Clark and Adams, 1981; Feltz and Trautmann, 1982; Chesnut, 1983), iontophoretic application of agonists (Dreyer et al., 1978; Pennefather and Quastel, 1982; Feltz and Trautmann, 1982), photoisomerizable agonists (Chabala et al., 1985, 1986), and steady-state single channel recording (Ogden and Colquhoun, 1983; Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986, 1987; Colquhoun and Ogden, 1988). In addition, a biochemical approach using rapid mixing and quenching methods and ion flux measurements has been reported (Udgaonkar and Hess, 1987a and b).

Here, we show how the question of the efficacy of acetylcholine can be addressed using a technique for the rapid perfusion of agonists to outside-out membrane patches (Brett et al., 1986). This approach offers a very direct measurement of efficacy, based on relatively few assumptions. In addition, the technique allows construction of a concentration-response curve over a large range of agonist concentrations, estimation of the number of channels in a patch and characterization of a fast desensitization process.

MATERIALS AND METHODS

For these studies we chose the BC3H-1 cell line (Schubert et al., 1974). The nicotinic acetylcholine receptors expressed by these cells have been extensively characterized in their electrophysiological (Sine and Steinbach, 1984a and b, 1986, 1987), biochemical (Boulter and Patrick, 1974; Patrick et al., 1977) and pharmacological (Sine and Taylor, 1979, 1980, 1981) properties. The cells (gift of A. Ritchie) were grown using the culture methods described by Sine and Steinbach (1984a).

For electrophysiological recording, culture medium was replaced with a solution containing (in millimolars) NaCl (150), KCl (5.6), CaCl₂ (1.8), MgCl₂ (1.0), and Hepes (10), pH 7.3, and the dish was placed in a plexiglass holder mounted on the stage of an inverted microscope. The patch pipette contained KCl (140), EGTA (5), MgCl₂ (5), and Hepes (10), pH 7.3. An outside-out membrane patch (Hamill et al., 1981) was excised from the cells and moved into position for rapid solution exchanges. The perfusion system consisted of a Y-shaped piece of tubing having two inflow tubes (connected to reservoirs) and one outflow tube immersed in the culture dish. The membrane patch was positioned within the outflow tube. A solenoid-driven pinch valve was used to

occlude one of the inflow tubes while releasing the other, permitting timed steps from agonist-free control solution to defined ACh concentrations and back. Under optimal conditions, this system allows the solution across an outside-out patch, and its associated unstirred layer, to be completely exchanged in 5 ms or less (Brett et al., 1986; cf. Fig. 1).

Currents were measured with a patch clamp amplifier (model EPC7, List-Electronic, Darmstadt, FRG), filtered at 2 kHz (eight-pole low-pass filter; Frequency Devices Inc., Haverhill, MA) digitized at 195 or 244 μ s per point (Sensorm, Burlington, VT) and stored on the hard disk of a laboratory minicomputer (Micro 11/73, Digital Electronics Corp., Maynard, MA). Data analysis was performed off-line with user-written programs.

The success rate of the rapid perfusion technique was limited to ~50% by a number of factors. Some otherwise satisfactory outside-out membrane patches did not survive the flowing solution or were subsequently lost on the first or second solution exchange pulse. In other cases, the speed of agonist perfusion was poor for no obvious reason. Many patches suffered an apparent loss of channels during the course of the experiment, which sometimes precluded comparison of the open channel probability associated with different ACh concentrations. The data presented here, then, were gathered from eight robust patches, which lasted from ~20 min to 2 h, exhibited little or no loss of channels over time, and for which the solution exchange rate was optimal.

The current response to a step application of ACh to a patch containing ACh receptor channels consists of a rapid onset followed by a slower decay. Because the number of active channels in these patches varied between 5 and 300, single channel analysis of these currents was usually precluded by the presence of many overlapping events. However, the decay phase of the responses is well suited to nonstationary fluctuation analysis (Sigworth, 1980). An ensemble of current responses is obtained by making 20–60 rapid applications of a given concentration of ACh. The ensemble mean and variance are calculated. If we assume that the measured currents arise from N independent, identical channels with a single channel amplitude i , and having a time-dependent open probability $p(t)$, we can write expressions for the mean current $I(t)$ and variance $\sigma^2(t)$ as:

$$I(t) = N i p(t) \quad (1)$$

$$\sigma^2(t) = N i^2 p(t) [1 - p(t)]. \quad (2)$$

We now eliminate $p(t)$ to obtain an expression relating the variance and the mean:

$$\sigma^2(t) = i I(t) - I^2(t)/N. \quad (3)$$

The free parameters of Eq. 3, i and N , can be determined by fitting the experimental data to a parabolic function. Because in these experiments it was possible to measure the single channel current directly (at low agonist concentrations when only a few channels were open simultaneously, or at high concentrations after most of the channels had desensitized, or when there were very few channels in the patch), we used a one-parameter curvilinear fitting routine to determine the number of channels from the variance vs. mean plot. The background variance (the variance before agonist application) was subtracted before fitting the data to Eq. 3.

The ensemble mean current was fitted to a single exponential function using a nonlinear routine which minimized chi squared using the Marquardt algorithm (Bevington, 1969). Three parameters were obtained from the fit: the initial and steady-state current values and a time constant. The steady-state current may not have been reached by the end of the agonist application period and is, therefore, an extrapolated rather than a measured value.

The typical protocol for an experiment was to make a series of

200–350-ms applications of 100 μ M ACh every 3–4 s. This was followed by a series of applications to one to three lower or higher concentrations and a return to 100 μ M.

Because the accuracy of the determination of N from the data is best when the fraction of open channels varies from a large value to near zero during agonist application, we usually calculated N from the 100- μ M data only. If the values of N calculated from two separate 100 μ M ACh runs were the same, this value of N was used for the intermediate runs at other concentrations. If the amplitude of the response decreased between the two 100- μ M ACh runs, the value of N for the intermediate runs was obtained by interpolation. The initial open channel probability, p_o , was calculated from the initial (peak) current using Eq. 1. When the ensemble mean current was smaller than a few picoamps (e.g., at ACh concentrations <3 μ M) we used an alternative means of calculating p_o . These records had clearly discernible single channel levels. We assumed that desensitization was negligible during agonist application (see Results) and calculated the open probability from the fraction of time that channels were open divided by the number of channels in the patch.

We estimated the time needed to effect a concentration change across an outside-out patch by examining the onset phase of the ensemble mean current response to ACh. This is illustrated in Fig. 1, where, for two different patches, we have superimposed the onset phases of the response of the patch to 100 or 30 μ M ACh (solid traces) and the response of the patch electrode (after the demise of the patch) to step changes in electrolyte concentration (dotted traces). The latter traces (solution exchange traces), show the speed of the perfusion system, which is determined by mixing and diffusion. The traces in the left panels of the figure were obtained from a patch under conditions where the solution exchange was moderately rapid (~2 ms, 10–90%); those in the right

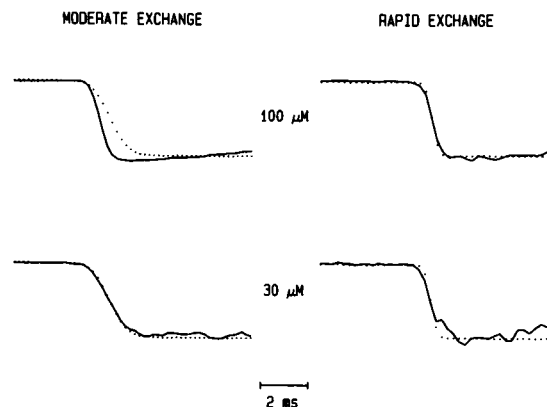


FIGURE 1 The onset of the current response of two outside-out patches to 100 and 30 μ M ACh (solid traces) compared to the onset of the "solution exchange responses" of the open patch electrodes during a change in electrolyte concentration from 150 to 75 mM NaCl (dotted traces). Ensemble means of 5–20 responses are shown. Current amplitudes are scaled to facilitate comparisons. The traces in the left panels came from an experiment in which the solution exchange had a risetime (time constant of a single exponential fit of the onset phase) of 0.7 ms (patch P10, sampling at 195 μ s/point); those on the right came from an experiment in which the solution exchange had a risetime of ~0.3 ms (patch P25, sampling at 244 μ s/point). Saturation and desensitization conspire to shorten the apparent response time to 100 μ M ACh (upper left). The best estimates of the speed of perfusion are made by considering subsaturating concentrations of agonist (30 μ M, lower panels).

panels came from a second patch for which the solution exchange was quite rapid (~ 1 ms). For the first patch, the onset of the response to $100 \mu\text{M}$ ACh (*upper left*) is faster than the onset of the solution exchange. However, this concentration of acetylcholine is in the saturating part of the concentration-response curve (see Results), so that the current reaches its maximum value before the solution exchange is complete. When $100 \mu\text{M}$ ACh was applied to the second patch (*upper right*), this effect was not apparent. At subsaturating concentrations of agonist ($30 \mu\text{M}$), however, the onset of the response to agonist is a good measure of the speed of solution exchange for both patches (two lower panels). Thus, in assessing the speed of the concentration change of any perfusion system, it is essential to choose concentrations of agonist which are below saturation yet high enough to avoid having the onset of the response be limited by kinetic activation steps. Furthermore, the presence of a rapid desensitization process may cause response times to be underestimated. These issues are not usually addressed when new methods for rapid perfusion are reported.

Channel block by ACh is a fast process (mean blocked time $< 25 \mu\text{s}$, Sine and Steinbach, 1984b; Ogden and Colquhoun, 1985) which, in the time resolution of our experiments, is manifest only as a reduction in single channel current. In these experiments, performed at room temperature (20 – 23°C) and at an applied potential of -50 mV, channel block should come into play only at ACh concentrations of $1,000 \mu\text{M}$. Because we measured the single channel current directly and used this value in the calculation of p_o , channel block should not affect our results.

Inward currents are shown as negative deflections in the figures.

RESULTS

Fig. 2 *a* shows an example of the current response obtained by exposing an outside-out patch from a BC3H-1 cell to a single application of $100 \mu\text{M}$ ACh for 250 ms. Seven or eight individual channel levels are readily seen in this record. Subsequent analysis of an ensemble of 60 such responses indicated the presence of eight activatable channels in this patch. Fig. 2 *b* represents the amplitude distribution of these 60 current responses to $100 \mu\text{M}$ ACh. The distribution shows that this patch contained only a single species of ACh receptor channel (as distinguished by channel amplitude). The average spacing of the peaks of the distribution is 1.95 pA. When cells were used within 1 wk of reducing the serum levels, we consistently saw only one channel amplitude in agreement with Sine and Steinbach (1984a).

Fig. 2 *c* is the ensemble mean current produced by 60 applications of $100 \mu\text{M}$ ACh to the same patch. Channel activation by $100 \mu\text{M}$ ACh is followed by a decay of the current with a time constant of 95 ms. Because it occurs while the patch is still exposed to agonist, this decay may be ascribed to a desensitization process. The effect of desensitization is dramatically illustrated in Fig. 3, showing a patch containing a large number of channels exposed to $100 \mu\text{M}$ ACh for 4 s. Here, the amplifier saturated when the current exceeded -95 pA (corresponding to 49 channels, -1.95 pA/channel) but on the basis of data obtained at a lower gain, we estimate that

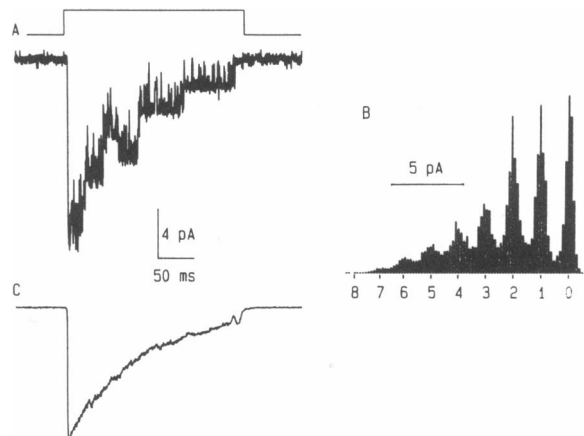


FIGURE 2 Results from the rapid perfusion of $100 \mu\text{M}$ ACh onto an outside-out patch of membrane from a BC3H-1 cell which contained relatively few channels (patch P4). (A) A single record of the current flowing through the patch as the agonist concentration is stepped from 0 to $100 \mu\text{M}$ and back. Single channel levels are clearly distinguished. Solid line at top of the figure shows the time course of the concentration change. (B) The amplitude histogram obtained from the ensemble of 60 records similar to the one in A. The peak on the right corresponds to times during which no channels were open. Times when the agonist concentration was zero were not included in the histogram. The figure shows the presence of eight channels each having a single channel current of -1.95 pA. (C) The ensemble mean current obtained from 60 rapid applications of $100 \mu\text{M}$ ACh to the patch. The time constant for the decay (95 ms) was obtained by fitting the mean current to a single exponential. The dip seen in the current trace near the end of agonist application is an artifact due to rapid perfusion. We have found that such artifacts can be eliminated by repositioning the patch electrode or adjusting the flow rate.

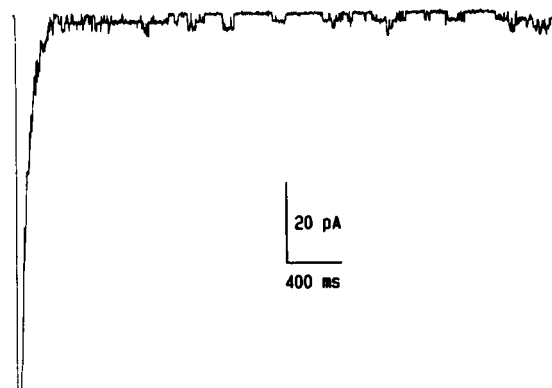


FIGURE 3 A record obtained during rapid application of $100 \mu\text{M}$ ACh to a patch containing ~ 150 channels. The duration of the application was 4 s and the sampling rate was 2 ms/point. The return to agonist-free control solution is not shown (patch P1-G4).

the patch contained ~ 150 channels. Because of this fast desensitization process, after the first 300 ms of agonist application, there are never more than three channels open simultaneously.

The concentration dependence of the response of a single outside-out patch is shown in Fig. 4. The ensemble mean currents from 56–60 applications of 3, 10, 30, and 100 μM ACh are superimposed. The peak current amplitude increases monotonically with agonist concentration. The relatively small increase in amplitude between 30 and 100 μM ACh suggests that the response is approaching saturation at 100 μM .

The time course of the current decay during the 250 ms exposure to agonist depends on agonist concentration; for Fig. 4, the decay time constants, τ , were >200 , 113, 110, and 84 ms for 3, 10, 30, and 100 μM ACh, respectively. In other experiments, using longer agonist applications, we found that this increase in τ with decreasing agonist concentration continued such that at 1 μM ACh, $\tau \approx 1$ s. The decay time constant was quite variable from patch to patch, the range for 100 μM ACh being 46–95 ms (see Table 1), and that, within the course of an experiment on a single patch, τ could vary by as much as 50%. In a few patches, we examined the voltage dependence of the current decay. Over a range of -100 to $+50$ mV, there was virtually no voltage dependence to the decay time constant of the response to 100 μM ACh (in one patch the time constants were 62, 49, and 63 ms for -100 , -50 , and 50 mV, respectively).

Although the duration of agonist application in these experiments was not long enough to determine the full extent of current decay (steady-state open channel probability), it does appear that this parameter is to some extent concentration-dependent. The steady-state current was 1–5% of the initial current for 10–1,000 μM ACh, but may be $\sim 50\%$ for lower agonist concentrations.

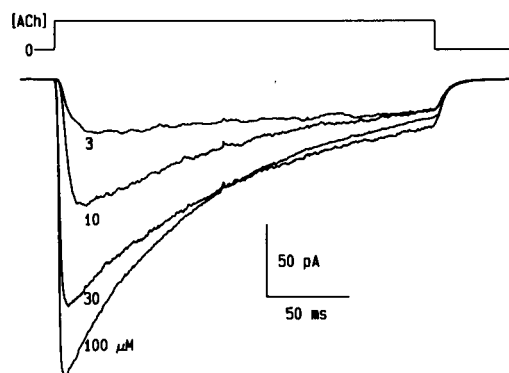


FIGURE 4 The ensemble mean current for four different concentrations of ACh on the same patch. An electrical artifact is seen at the time when the solenoid-driven valve was switched off (patch D1).

TABLE 1 Measured and calculated parameters for individual patches

Patch	N_{\max}	τ	p_{\max}	p_{\max}^*
		ms		
D1	107	84	0.95	1.03
P3	156	58	0.81	0.89
P4	10	95	0.84	0.88
P10	44	55	0.89	0.91
P15	306	76	0.99	1.05
P16	40	46	0.80	0.84
P20	16	70	0.93	0.95
P25	5	51	0.92	0.93

For the eight patches considered in this paper, we show the maximum number of channels observed in the patch (N_{\max}) determined by nonstationary fluctuation analysis (there may have been some run-down during the experiment), the time constant (τ) for the decay of the response at 100 μM ACh, the maximum open channel probability p_{\max} (the value of p_0 for 100 μM ACh) and the corrected open channel probability, p_{\max}^* , based on the speed of the perfusion system and desensitization (see Discussion).

The results of ensemble fluctuation analyses of the data in Fig. 4 are presented in Fig. 5. The results from all four concentrations are shown on a single variance vs. mean curve. A single channel current of -2.0 pA was obtained from an analysis of individual current records. A one-parameter curvilinear regression analysis was used to determine the best value of N to fit the data. Analysis of both the 100 and 30 μM data gave $N = 107 \pm 5$. The open channel probability did not reach high enough values at the lower concentrations to determine N with any precision. However, visual inspection of Fig. 5 indicates that the parabola generated by $N = 107$ is a reasonable fit for

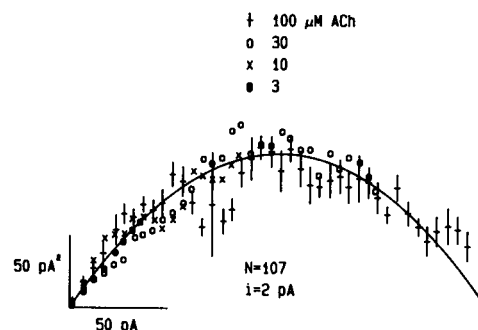


FIGURE 5 The ensemble variance vs. mean curves for the data of Fig. 4. The parabola was drawn according to Eq. 3 with $N = 107$ and $|i| = 2.0$ pA. The background variance was 0.15 pA^2 . Each point was obtained by grouping the values of ensemble mean current (I) into 5 pA bins and calculating the average of the associated values of variance (σ^2). For the 100 μM data we also show the standard deviations of the average values of variance. The absolute values of the mean current have been used in this figure only.

all four concentrations. Estimates of the initial probability, p_0 , associated with each ACh concentration are obtained directly from Eq. 1, using the peak value of I . For the patch illustrated in Fig. 5, p_0 is 0.18 at 3 μM , 0.41 at 10 μM , 0.73 at 30 μM , and 0.95 at 100 μM ACh.

In these experiments, the number of activatable channels in outside-out patches from BC3H-1 cells ranged from 5 to >300 (Table 1). If we include patches which could not be fully analyzed (for the reasons discussed under Methods), the number of channels in the patch was as high as 550, but generally ranged between 50 and 150.

The acetylcholine concentration-response curve, using data obtained from eight membrane patches is shown in Fig. 6. In Fig. 6 *a*, the initial open channel probability is plotted as a function of the logarithm of the agonist concentration. A concentration of 20 μM ACh activates half the channels and the maximum open channel probability is ≥ 0.90 . When $\log p_0$ is plotted vs. $\log [\text{ACh}]$, as in Fig. 6 *b*, the slope of the curve at low concentrations is 1.95.

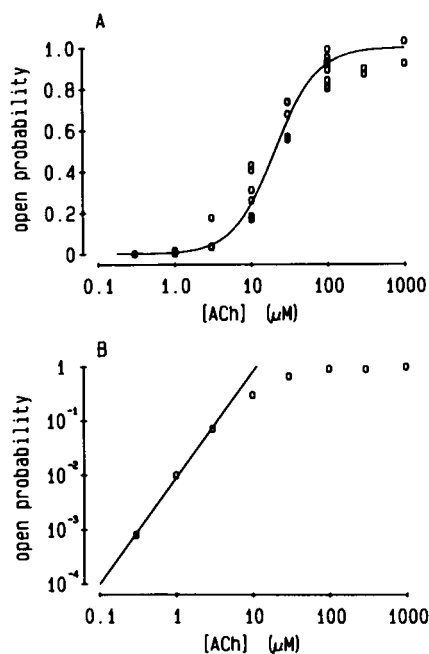


FIGURE 6 Concentration-response relationship for ACh on the nicotinic ACh receptor. (A) The initial open channel probability as a function of $\log[\text{ACh}]$. Each data point represents a separate series of agonist applications to one of the eight patches considered in this paper (2, 3, 4, 6, 5, 8, 2, and 2 series at 0.3, 1, 3, 10, 30, 100, 300, and 1,000 μM ACh). Solid line superimposed on the data is the best fit (nonlinear least squares) to the Hill equation $p = 1/[1 + (K_m/[\text{ACh}])^n]$ with the half-maximal concentration, $K_m = 20 \mu\text{M}$ and Hill coefficient $n = 1.6$. (B) Log-log plot of the data expressed as the mean value of p at each concentration. Linear regression of the data for $\leq 3 \mu\text{M}$ ACh produced a best fit line of slope 1.95.

DISCUSSION

We have demonstrated how a number of the properties of nicotinic acetylcholine receptor ion channels can be directly measured using our method of patch clamp recording from excised patches during rapid perfusion of agonists. To a certain extent, these properties have been deduced from indirect measurements by others. In this section, we discuss our findings and compare them to those obtained using other techniques.

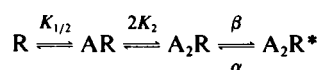
Since single channel recording techniques were first used to study the nicotinic ACh receptor (Neher and Sakmann, 1976), it has been noted that the maximum number of channels which could be activated by high concentrations of nicotinic agonists is considerably lower than estimates of channel density would predict. The usual interpretation of this observation is that most of the channels become desensitized during the process of seal formation (with a patch electrode containing ACh) and that recovery from desensitization is slow (Sakmann et al., 1980). The alternative explanations are that even high concentrations of agonist are unable to open a large fraction of the available channels or that the channels are irreversibly damaged during seal formation (a frightening prospect for patch clampers in general). Here we show that the usual interpretation is probably correct, at least for receptors in BC3H-1 cells. Only when a high concentration of agonist is perfused to the patch within milliseconds are most of the channels simultaneously open.

We find that outside-out patches from BC3H-1 cells contain, typically, 50–150 functional agonist-activated channels. This estimate is based on the peak current produced during rapid perfusion of high concentrations of ACh. Interestingly, Sine and Steinbach (1986) obtained similar values from their single channel studies by considering the burst frequency at low agonist concentrations and their determination of kinetic rate constants. In light of the possibility that not all ACh receptors are functional (Catterall, 1975; Jackson, 1988), it is tempting to convert these numbers to channel density and to compare this with an estimate of receptor density of $73/\mu\text{m}^2$ derived from α -bungarotoxin binding studies (Rosenthal, J., and M. M. Salpeter, as quoted in Papke et al., 1988). The surface area of outside-out patches with resistances of 3–6 $\text{M}\Omega$ apparently varies considerably (range 1–14 μm^2 , Sakmann and Neher, 1983). Thus, our average number of functional channels per patch of 86 implies a channel density on the order of 10–100/ μm^2 , which is consistent with the receptor density.

Acetylcholine is an efficacious agonist. At 100 μM ACh, a concentration used on all of the patches and near the saturating part of the concentration-response curve, the average maximum open channel probability, p_{max} , is

0.89 ± 0.07 (Table 1).¹ This value represents a lower limit on p_{\max} because some desensitization occurs before the concentration change is complete. A rough estimate of the magnitude of this effect can be obtained by multiplying p_{\max} by $\exp(\tau_{\text{onset}}/\tau)$, where τ_{onset} is the time constant for the onset phase of the response at subsaturating agonist concentrations (e.g., as in Fig. 1) and τ is the decay time constant at 100 μM . These corrected values, p_{\max}^* are listed in Table 1. The average value of p_{\max}^* is 0.93, an increase of 5% over the uncorrected values. Because the actual form of the correction factor requires detailed information about the solution exchange profile and a priori knowledge of the concentration dependence of p_o , we prefer to use the uncorrected values of p_o and simply present them as lower limits.

The only assumptions inherent to the determination of p_{\max} from nonstationary fluctuation analysis are those of channel independence and uniformity. In contrast, estimates of p_{\max} from analyses of steady-state single channel records require certain assumptions about ACh receptor channel activation schemes (Ogden and Colquhoun, 1983; Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986, 1987; Auerbach and Lingle, 1986; Hestrin et al., 1987; Colquhoun and Ogden, 1988). Specifically, two approaches have been taken. Both are based on the observation that ACh receptor channel activity tends to occur in "bursts"—several closely spaced openings, the gaps between openings being 1 ms or less—separated by long closed periods. They are also based on the following kinetic reaction scheme:



In this scheme, two agonist molecules (A) sequentially bind to two equivalent sites on the receptor (R) (equilibrium binding constants K_1 and K_2) to form a doubly-liganded closed state (A_2R). This state then undergoes isomerization to an open state (A_2R^*) at a rate β , which may subsequently close at a rate α . In addition, one or more desensitized (closed) states are thought to be connected to the other states in some yet unknown way.

In the first approach, bursts of channel activity seen at low concentrations of agonist are analyzed. The kinetic scheme predicts that bursts of channel activity arise from the repeated opening of a single channel in the A_2R state before an agonist molecule dissociates from the receptor. Under this assumption, values for α and β can be calculated from measurements of the duration of openings and the frequency and duration of the short gaps within

bursts. Efficacy is then calculated from $\beta/(\alpha + \beta)$. The uncertainty in this approach arises from the observation that bursts are composed of at least two distinct populations of gaps, only one of which can actually correspond to the isomerization transitions (Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986). For BC3H-1 cells with ACh as agonist, Sine and Steinbach (1986) found that $\beta/(\alpha + \beta)$ was either 0.93 or 0.995, depending on which population of gaps was considered to be associated with isomerizations. They preferred the former value, however, after analyzing burst kinetics with other agonists as well. Their selection criterion, that the isomerization transitions should occur at different rates for different agonists (agonist specificity), is arbitrary. Interestingly, using the same criterion, Colquhoun and Sakmann (1985) found that for frog neuromuscular junction, the larger of two possible values of $\beta/(\alpha + \beta)$, 0.98, was preferred.

The second approach is to consider steady-state single channel data at saturating concentrations of agonist ($\geq 100 \mu\text{M}$ ACh). These records contain short periods of single channel activity separated by long periods of silence. The assumption is made that the long periods of inactivity represent times when all of the channels are desensitized. These periods are ignored and the distribution of the remaining shorter closed times is considered. If it is possible to identify the components of this distribution which correspond to the binding and opening reactions, efficacy can be calculated from the fraction of time the channel is open during a single group of openings. The multitude of closed-time components is again a confounding problem: three or four components are often found. In their experiments with frog neuromuscular junction, Ogden and Colquhoun (1983) chose the two shortest components and calculated an efficacy of 0.98, in agreement with what Colquhoun and Sakmann (1985) later found using the low concentration approach. Sine and Steinbach present an argument for their selection of the appropriate closed-time components for ACh receptors in BC3H-1 cells, but it is unsettling that the origins of the remaining components remain unknown. Nevertheless, their result, $p_{\max} = 0.91$, is also consistent with their low agonist concentration results.

Sine and Steinbach (1987) also determined the agonist concentration dependence of p_o in BC3H-1 cells using the fractional open time method described above. The method is not useful for concentrations of ACh below 20 μM because it becomes too difficult to distinguish the endpoints of groups. At 20 μM ACh, however, they calculated a value of 0.7–0.8 for p_o , implying that an open channel probability of 50% is reached at lower concentrations. When they used their values of all of the rate constants in the kinetic scheme, they found $K_m = 7 \mu\text{M}$ at 11°C, a bit lower than our value of 20 μM at 21°C. Using

¹Our determination of p_{\max} is actually the product of maximum binding and efficacy. Thus, 0.89 represents a lower limit on efficacy.

the same type of analysis, Sakmann et al. (1980) obtained $K_m = 20 \mu\text{M}$ for frog muscle.

Other methods which have given estimates of the K_m for ACh include "slow" perfusion by Adams and Feltz (1980) ($K_m = 20 \mu\text{M}$) quantitative iontophoresis (Dreyer et al., 1978) ($K_m = 20 \mu\text{M}$) and rapid mixing/ion flux experiments (Forman and Miller, 1988) ($K_m = 100 \mu\text{M}$). Adams and Feltz (1980) also estimated the efficacy of ACh to be 0.70 but neither of the other two techniques provide an absolute measure of efficacy.

We were able to extend our measurements of open channel probability down to $0.3 \mu\text{M}$ ACh. The shape of the low concentration portion of the curve gives an indication of the number of agonist molecules needed to open the channel. As seen in Fig. 6 *b*, the low concentration portion of the curve varies as $[\text{ACh}]^{1.95}$. This essentially quadratic dependence on concentration is consistent with models in which two molecules of ACh are needed to open the channel (Adams, 1975; Lester et al., 1975; Dionne et al., 1978; Pennefather and Quastel, 1982; Trautmann, 1983). It has been noted, however, that it is possible to ascribe some portion (at concentrations $< 200 \text{ nM}$ ACh) of the brief channel openings in BC3H-1 receptors to the opening of monoliganded receptor channels (Papke and Oswald, 1986; our unpublished results). The existence of a monoliganded brief open state would tend to reduce the slope of the $\log p_o$ vs. $\log [\text{ACh}]$ curve at low agonist concentrations.

The concentration-response data we obtained using the model-independent approach described here may also be fitted to the conventional kinetic scheme (shown above) to obtain estimates of binding affinities and efficacy. Assuming that the two binding sites do not interact (no cooperativity), we find that $K_1 = K_2 = 56 \mu\text{M}$ and $\beta/\alpha = 17$. Although the degree of cooperativity can often be determined from the slope of the concentration-response curve, the confidence intervals are wide or even indeterminate for the case of an efficacious agonist such as ACh (Colquhoun and Ogden, 1988). We obtained no significant improvement in the fit by allowing K_1 and K_2 to vary freely.

Some information about the kinetics of desensitization can be derived from the current decay that is observed after rapid application of agonists. First, we note that the time constant of the decay, τ , appears to be dependent on agonist concentration. One simple kinetic scheme which would predict this dependence is one in which channels must open before they can desensitize. In this model, if the initial current arises from the activation of most of the channels ($[\text{ACh}] \geq 100 \mu\text{M}$), τ is approximately $(d_+ + d_-)^{-1}$ where d_+ and d_- are the rate constants for entering and leaving the desensitized state, respectively. The ratio of steady-state current to initial current in this scheme, is proportional to d_- / d_+ . For high agonist concentrations,

this ratio is < 0.1 ; so $d_+ > d_-$ and $d_+ \approx 1/\tau = 10\text{--}20/\text{s}$.² A more detailed analysis of desensitization will be possible only after performing experiments with longer agonist applications. However, assessing the kinetics of desensitization from rapid agonist application is quite straightforward because the observed currents are essentially "macroscopic" in amplitude. Making such measurements will facilitate the identification of desensitization processes with components of closed-time histograms derived from single channel recordings. In addition, determination of the number of channels in the patch will clarify the interpretation of the longest components of closed-time histograms.

We have compared our values of time constants with other reported values. Although these measurements were made with a variety of receptor preparations, temperatures, and agonists (ACh or carbachol), it seems that the technique used is the most important variable. When agonists are perfused to the preparation relatively slowly (Clark and Adams, 1981; Feltz and Trautmann, 1982; Chesnut, 1983; Adams, 1987), desensitization appears on the time scale of seconds, similar to the time scale of the concentration change. With iontophoretic applications of agonist, desensitization time constants either on the order of seconds (Pennefather and Quastel, 1982; Feltz and Trautmann, 1982; Trautmann, 1983) or tens to hundreds of milliseconds (Dreyer et al., 1978; Anwyl and Narahashi, 1980) have been observed. Although iontophoretic application is rapid, the agonist concentration is not well known (but see Dreyer et al., 1978). Using nerve-released ACh, Magleby and Pallotta (1981) found evidence for a desensitization process occurring on the tens of milliseconds time scale. Rapid chemical kinetic techniques, which measure the flux of labeled ions through ACh receptors in vesicles after short (greater than several milliseconds) exposures to agonists, have also revealed fast processes taking place within tens to hundreds of milliseconds (Udgaonkar and Hess, 1987*a* and *b*; Forman and Miller, 1988).

There have been reports that desensitization proceeds with a voltage-dependent time course (Magazanik and Vyskocil, 1970; Scubon-Mulieri and Parsons, 1978; Magleby and Pallotta, 1981). The component we observe shows no voltage dependence over the range of -100 to $+50 \text{ mV}$. In their single channel experiments using frog muscle, Colquhoun and Ogden (1988) saw little or no voltage dependence in desensitization rates.

Among the most novel technical approaches to making rapid changes in agonist concentration is the use of

²Direct measurement of the rate of recovery from desensitization (our unpublished results) confirm that recovery is a slower process than desensitization itself, $d_- \approx 2/\text{s}$.

photochemically activated agonists (Chabala et al., 1985, 1986). One isomer of the azobenzene derivative Bis-Q acts as an agonist for the nicotinic ACh receptor, a second isomer is inactive. The inactive form is converted to the active form by exposure to a light flash. Chabala et al. (1985, 1986) investigated the low concentration part of the concentration-response curve for Bis-Q and found an initial slope of two. No estimates of efficacy or K_m could be made. Naturally, this technique is limited to the few agonists which have photochemical properties; ACh is not among them.

The rapid perfusion technique has several limitations. Although the speed of the concentration change around a membrane patch is diffusion limited, it is not fast enough to entirely avoid desensitization before the exchange is complete. Also, it is not fast enough to allow the determination of rate constants from the onset phase of the response (the neuromuscular junction itself has an order of magnitude advantage here). We are disturbed by the apparent loss of channels over time seen in some patches and the variability in the kinetics of desensitization. These last two problems may be a consequence of patch excision and might be remedied by the inclusion of some cytosolic component in the patch electrode solution.

Despite these limitations, the technique has allowed us to generate a quantitative dose-response curve for acetylcholine, essentially free of the assumptions and extrapolations which have characterized this effort in the past. In the course of these experiments, the shape of the dose-response curve, the density of acetylcholine receptor channels in BC3H-1 cells, and several properties of a rapid desensitization process have been determined.

The authors thank Dr. Paul R. Adams for the impetus to attempt these experiments, for encouragement and advice, and for the use of facilities during the initial part of the project.

This work was supported by the National Institutes of Health (grant NS21581 to J. P. Dilger).

Received for publication 3 May 1989 and in final form 19 October 1989.

REFERENCES

- Adams, P. R. 1975. An analysis of the dose-response curve at voltage-clamped frog-endplates. *Pfluegers Arch. Eur. J. Physiol.* 360:145-153.
- Adams, P. R. 1987. The Vertebrate Neuromuscular Junction. M. M. Salpeter, editor. Alan R. Liss, Inc., New York. 317-359.
- Adams, P. R., and A. Feltz. 1980. End-plate channel opening and the kinetics of quinacrine (mepacrine) block. *J. Physiol. (Lond.)* 306:283-306.
- Anwyl, R., and T. Narahashi. 1980. Desensitization of the acetylcholine receptor of denervated rat soleus muscle and the effect of calcium. *Br. J. Pharmacol.* 69:91-98.
- Auerbach, A., and C. J. Lingle. 1986. Heterogeneous kinetic properties of acetylcholine receptor channels in *Xenopus* myocytes. *J. Physiol. (Lond.)* 378:119-140.
- Beverington, P. R. 1969. Data reduction and error analysis for the physical sciences. McGraw-Hill Book Co., New York.
- Boulter, J., and J. Patrick. 1977. Purification of an acetylcholine receptor from a nonfusing muscle cell line. *Biochemistry*. 16:4900-4908.
- Brett, R. S., J. P. Dilger, P. R. Adams, and B. Lancaster. 1986. A method for the rapid exchange of solutions bathing excised membrane patches. *Biophys. J.* 50:987-992.
- Catterall, W. A. 1975. Sodium transport by the acetylcholine receptor of cultured muscle cells. *J. Biol. Chem.* 250:1776-1781.
- Chabala, L. D., A. M. Gurney, and H. A. Lester. 1985. Photoactivation and dissociation of agonist molecules at the nicotinic acetylcholine receptor in voltage-clamped rat myoballs. *Biophys. J.* 48:241-246.
- Chabala, L. D., A. M. Gurney, and H. A. Lester. 1986. Dose-response of acetylcholine receptor channels opened by a flash-activated agonist in voltage-clamped rat myoballs. *J. Physiol. (Lond.)* 371:407-433.
- Chesnut, T. J. 1983. Two-component desensitization at the neuromuscular junction of the frog. *J. Physiol. (Lond.)* 336:229-241.
- Clark, R. B., and P. R. Adams. 1981. Rapid flow measurements of desensitization at frog endplates. *Biophys. J.* 33:16a. (Abstr.)
- Colquhoun, D., and D. C. Ogden. 1988. Activation of ion channels in the frog end-plate by high concentrations of acetylcholine. *J. Physiol. (Lond.)* 395:131-159.
- Colquhoun, D., and B. Sakmann. 1985. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. *J. Physiol. (Lond.)* 369:501-557.
- Dionne, V. E., J. H. Steinbach, and C. F. Stevens. 1978. An analysis of the dose-response relationship at voltage-clamped frog neuromuscular junctions. *J. Physiol. (Lond.)* 281:421-444.
- Dreyer, F., K. Peper, and R. Sterz. 1978. Determination of dose-response curves by quantitative iontophoresis at the frog neuromuscular junction. *J. Physiol. (Lond.)* 281:395-419.
- Feltz, A., and A. Trautmann. 1982. Desensitization at the frog neuromuscular junction: a biphasic process. *J. Physiol. (Lond.)* 322:257-272.
- Forman, S. A., and K. W. Miller. 1988. High acetylcholine concentrations cause rapid inactivation before fast desensitization in nicotinic acetylcholine receptors from *Torpedo*. *Biophys. J.* 54:149-158.
- Hamill, O. P., E. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch. Eur. J. Physiol.* 391:85-100.
- Hestrin, S., J. I. Korenbrot, and A. V. Maricq. 1987. Kinetics of activation of acetylcholine receptors in a mouse cell line under a range of acetylcholine concentrations. *Biophys. J.* 51:449-455.
- Jackson, M. B. 1988. Dependence of acetylcholine receptor channel kinetics on agonist concentration in cultured mouse muscle fibres. *J. Physiol. (Lond.)* 397:555-583.
- Katz, B., and S. Thesleff. 1957. A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol. (Lond.)* 138:63-80.
- Lester, H. A., J.-P. Changeux, and R. E. Sheridan. 1975. Conductance increases produced by bath application of cholinergic agonists to *Electrophorus* electroplaques. *J. Gen. Physiol.* 65:797-816.
- Magazanik, L. G., and F. Vyskocil. 1970. Dependence of acetylcholine

- desensitization on the membrane potential of frog muscle fibre and on the ionic changes in the medium. *J. Physiol. (Lond.)*. 210:507-518.
- Magleby, K. L., and B. S. Pallotta. 1981. A study of desensitization of acetylcholine receptors using nerve-released transmitter in the frog. *J. Physiol. (Lond.)*. 316:225-250.
- Neher, E., and B. Sakmann. 1976. Single channel currents recorded from membrane of denervated frog muscle fibres. *Nature (Lond.)*. 260:799-802.
- Ogden, D. C., and D. Colquhoun. 1983. The efficacy of agonists at the frog neuromuscular junction studied with single channel recording. *Pfluegers Arch. Eur. J. Physiol.* 399:246-248.
- Ogden, D. C., and D. Colquhoun. 1985. Ion channel block by acetylcholine, carbachol and suberyldicholine at the frog neuromuscular junction. *Proc. R. Soc. Lond. B Biol. Sci.* 225:329-355.
- Papke, R. L., and R. E. Oswald. 1986. Effects of allosteric ligands on the gating of single channel currents in BC3H-1 cells. In *Nicotinic Acetylcholine Receptor*. A. Maelicke, editor. Springer-Verlag, Berlin. 243-257.
- Papke, R. L., G. Millhauser, Z. Lieberman, and R. E. Oswald. 1988. Relationships of agonist properties to the single channel kinetics of nicotinic acetylcholine receptors. *Biophys. J.* 53:1-10.
- Patrick, J., J. McMillan, H. Wolfson, and J. C. O'Brien. 1977. Acetylcholine receptor metabolism in a nonfusing muscle cell line. *J. Biol. Chem.* 252:2143-2153.
- Pennefather, P., and D. M. J. Quastel. 1982. Fast desensitization of the nicotinic receptor at the mouse neuromuscular junction. *Br. J. Pharmacol.* 77:395-404.
- Sakmann, B., and E. Neher. 1983. Geometric parameters of pipettes and membrane patches. In *Single-Channel recording*. B. Sakmann and E. Neher, editors. Plenum Press, New York. 37-51.
- Sakmann, B., J. Patlak, and E. Neher. 1980. Single acetylcholine-activated channels show burst-kinetics in the presence of desensitizing concentrations of agonist. *Nature (Lond.)*. 286:71-73.
- Schubert, D., A. J. Harris, C. E. Devine, and S. F. Heinemann. 1974. Characterization of a unique muscle cell line. *J. Cell Biol.* 61:398-413.
- Scubon-Mulieri, B., and R. L. Parsons. 1978. Desensitization onset and recovery at the potassium-depolarized frog neuromuscular junction are voltage sensitive. *J. Gen. Physiol.* 71:285-299.
- Sigworth, F. J. 1980. The variance of sodium current fluctuations at the node of Ranvier. *J. Physiol. (Lond.)*. 307:97-129.
- Sine, S. M., and J. H. Steinbach. 1984a. Activation of a nicotinic acetylcholine receptor. *Biophys. J.* 45:175-185.
- Sine, S. M., and J. H. Steinbach. 1984b. Agonists block currents through acetylcholine receptor channels. *Biophys. J.* 46:277-284.
- Sine, S. M., and J. H. Steinbach. 1986. Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by low concentrations of agonist. *J. Physiol. (Lond.)*. 373:129-162.
- Sine, S. M., and J. H. Steinbach. 1987. Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by high concentrations of agonist. *J. Physiol. (Lond.)*. 385:325-359.
- Sine, S. M., and P. Taylor. 1979. Functional consequences of agonist-mediated state transitions in the cholinergic receptor. Studies on cultured muscle cells. *J. Biol. Chem.* 254:3315-3325.
- Sine, S. M., and P. Taylor. 1980. The relationship between agonist occupation and the permeability response of the cholinergic receptor revealed by bound cobra α -toxin. *J. Biol. Chem.* 255:10144-10156.
- Sine, S. M., and P. Taylor. 1981. The relationship between reversible antagonist occupancy and the functional capacity of the acetylcholine receptor. *J. Biol. Chem.* 256:6692-6699.
- Stephenson, R. P. 1956. A modification of receptor theory. *Br. J. Pharmacol.* 11:379-393.
- Trautmann, A. 1983. A comparative study of the activation of the cholinergic receptor by various agonists. *Proc. R. Soc. Lond. B Biol. Sci.* 218:241-251.
- Udgaonkar, J. B., and G. P. Hess. 1987a. Acetylcholine receptor: channel opening kinetics evaluated by rapid chemical kinetic and single channel current measurements. *Biophys. J.* 52:873-883.
- Udgaonkar, J. B., and G. P. Hess. 1987b. Chemical kinetic measurements of a mammalian acetylcholine receptor by a fast-reaction technique. *Proc. Natl. Acad. Sci. USA*. 84:8758-8762.