Opening rate of acetylcholine receptor channels

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ABSTRACT The nicotinic acetylcholine (ACh) receptor is responsible for rapid conversion of chemical signals to electrical signals at the neuromuscular junction. Because the receptor and its ion channel are components of a single transmembrane protein, the time between ACh binding and channel opening can be minimized. To determine just how quickly the channel opens, we made rapid (100–400 μs) applications of 0.1–10 mM ACh to outside-out, multichannel membrane patches from BC3H-1 cells, while measuring the onset of current flow through the channels at 11°C. Onset time is steeply dependent upon ACh concentration when channel activation is limited by binding of ACh (0.1–1 mM). At +50 mV, the 20–80% onset time reaches a plateau near 110 μs above 5 mM ACh as channel opening becomes rate limiting. Thus, we calculate the opening rate, $\beta \approx 12$/ms, without reference to specific channel activation schemes. At −50 mV, the combination of a rapid, voltage-dependent block of channels by ACh with a finite solution exchange time distorts onset. To determine opening rate at −50 mV, we determine the kinetic parameters of block from "steady-state" current and noise analyses, assume a sequential model of channel activation/block, and numerically simulate current responses to rapid perfusion of ACh. Using this approach, we find $\beta \approx 15$/ms. In contrast to the channel closing rate, the opening rate is relatively insensitive to voltage.

INTRODUCTION

The opening and closing rates of ligand-gated ion channels are not well known. Estimates of the opening rate of nicotinic ACh receptor channels, are based on several types of measurements: steady-state noise analysis (Sakmann and Adams, 1978), miniature end-plate current onset time (Land et al., 1981, 1984; Dwyer, 1981), and single channel kinetics (Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986, 1987). There is some confusion regarding the time scale of channel opening after ACh binding, estimates range from 20 μs to 3 ms. All of these measurements rely on assumptions about channel activation and one method, measurement of end-plate current onset time, also depends on modeling or transmitter release, diffusion processes, and synapse geometry (Land et al., 1980, 1981, 1984; Dwyer, 1981).

In principle, a direct method of determining the opening rate of ligand-gated ion channels is available through a combination of the patch clamp recording technique (Hamill et al., 1981) and a system for making rapid changes of solution around an excised patch (Brett et al., 1986; Maconochie and Knight, 1989; Dudel et al., 1990). Thus, analogous to rapid voltage-clamping of voltage-gated ion channels, the opening rate could be determined from the onset of current after rapid application of a super-saturating concentration of agonist. This would minimize the time needed for the concentration-dependent steps of channel activation and reveal the rate limiting, concentration-independent process. In practice, the multiple actions of agonists on ligand-gated channels (activation, desensitization, and channel block in the case of nicotinic ACh receptors) may limit the usefulness of this approach. In this report, we investigate the extent to which the opening rate of ACh receptor channels can be determined using an improved rapid perfusion technique.

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METHODS

Clonal BC3H-1 cells (Schubert et al., 1974) expressing nicotinic ACh receptors were cultured for electrophysiological experiments. Culture medium was replaced with extracellular solution (concentrations in millimolar: 150 NaCl, 5.6 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, and 10 Hepes, pH 7.3). Patch pipettes were fabricated from borosilicate glass, fire polished, coated with enamel paint, and filled with 140 KCl, 5 EGTA, 5 $MGCl_2$, and 10 Hepes, pH 7.3. A seal of >10 GΩ was established on-cell and the patch was excised in the outside-out configuration (Hamill et al., 1981). The patch was inserted into the outflow limb of a rapid perfusion apparatus consisting of reservoirs containing extracellular solution (with and without ACh), ethyl vinyl acetate tubing, and a solenoid-driven pinch valve. Changes from a previous design (Brett et al., 1986) include use of smaller gauge tubing (inside diameter 0.3 vs. 1.3 mm), higher voltage applied to the solenoid (24 vs. 12 V), and a different plumbing geometry. The outflow tubes were joined at a ≈50°

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angle and the patch was positioned symmetrically, <1 mm away from each tube (where streams coming from both tubes would have their confluence). Perfusion solutions were cooled to 10–12°C. The linear flow speed was ≈10 cm/s. The solution bathing the patch was rapidly changed from agonist-free solution to 0.1–10 mM ACh by switching the pinch valve and the current flowing through the patch was measured with a patch clamp amplifier. To minimize the effects of desensitization, the duration of exposure to ACh was limited to 20 ms and exposures were separated by 1 s.

About 50% of the patches obtained during this study had onset times >200 μs for applications of 0.1 mM ACh at 20–22°C. For two reasons, we consider these patches to be too slow to provide useful information about the channel opening rate. First, the concentrations of ACh which would be required to demonstrate saturation of onset time also produce significant channel block at +50 mV. Second, it becomes difficult to distinguish the decay due to block and the decay due to desensitization when the solution exchange occurs on the millisecond time scale. Data from "slow" patches are not included in the results presented in this paper. It may be that the surfaces of slow patches are not smooth but contain invaginations into which ACh diffuses slowly. Accumulation of debris at the tip of the patch pipette also leads to slower solution exchanges.

Currents were low-pass filtered at 10, 20, or 30 kHz (eight-pole Bessel filter), digitized at 3 μs/point and stored on the hard disk of a laboratory minicomputer. Sometimes, the raw data were refiltered digitally with a Gaussian filter (Colquhoun and Sigworth, 1983) to a final frequency of 10 kHz. This reduced the noise level without perturbing onset times > ~60 μs. Individual records were aligned with each other at the midpoint of the onset phase and the ensemble mean of 10–50 records was calculated. Onset curves are best fit by a sigmoidal function. The time constant of this function, however, does not have any obvious relationship to the channel opening rate. In the absence of channel block, the region of the onset curve >20% of maximum is well described by a single exponential function. We assume that this portion of the curve is determined primarily by the channel opening rate rather than agonist binding so that the time constant of the exponential function approximates the reciprocal of the opening rate. We tested this assumption by performing simulations as described below. Under the least favorable conditions relevant to this study (+50 mV, 5 mM ACh, and a 400 μs solution exchange time), the time constant of the fitted exponential underestimates the opening rate by 20%. When channel block was apparent, onset curves were not well fitted by a single exponential function. To compare these data with results of simulations, we define onset time, t_{onset}, as the 20–80% rise time. To avoid confusion, we use the same definition of onset time throughout the paper. Thus, at positive potentials, onset time divided by 1.39 gives the time needed for an e-fold change in current.

For the determination of the equilibrium blocking constant, K_B, the steady-state currents elicited by application of blocking concentrations of ACh, $I([ACh])$, were compared to the peak current elicited by 0.1 mM ACh, $I(0.1)$. The current for 0.1 mM ACh was measured both before and after each higher [ACh] series of applications to guard against errors due to any change in the number of active channels in the patch (Dilger and Brett, 1990). Block by ACh is assumed to be described by a sequential open channel blocking mechanism so that:

$$I([ACh])/I(0.1) = 1/(1 + [ACh]/K_B). \quad (1)$$

Eq. 1 is valid when the peak open probability for 0.1 mM ACh is close to one (Dilger and Brett, 1990). We obtain K_B from the slope of a plot of $I(0.1)/I([ACh])$ vs. [ACh].

We estimated that forward and reverse rate constants of fast block using stationary noise analysis (Ogden and Colquhoun, 1985). Currents were low-pass filtered at 30 kHz with an eight-pole Butterworth filter and digitized at 17 μs/point. Variance was calculated for individual records and averaged over 20–30 records. The measured variance, σ^2_m, arising from channel block was calculated by subtracting the variance of a 6 ms segment before agonist perfusion (background) from the variance of a 6 ms segment during agonist perfusion after the current had reached a steady level. The expected variance, σ^2_e was calculated from the steady-state ensemble mean current and the single channel current, i.

$$\sigma^2_e = I([ACh])i(1 - p), \quad (2)$$

where $p = (1+[ACh]/K_B)^{-1}$. For fast block, the measured variance will be less than the expected variance due to low-pass filtering. The ratio of expected to measured variance is related to the half-power frequency, f_o, of the process and the cutoff frequency of the filter. We used the equations derived by Ogden and Colquhoun (1985), which assume a Lorentzian spectrum for the blocking process and an eight-pole Butterworth filter to infer the half-power frequency of fast block by ACh. The forward, k_{+b}, and reverse, k_{-b}, blocking rates are then obtained from $K_B = k_{-b}/k_{+b}$ and $2\pi f_o = k_{+b}[ACh] + k_{-b}$.

Numerical integration of the differential equations describing Scheme II was used to generate computer simulations of onset currents. The agonist concentration was considered to have an exponential time-dependence:

$$[ACh](t) = [ACh]_a[1 - \exp(-t/\tau_s)], \quad (3)$$

where $[ACh]_a$ is the applied concentration of ACh and τ_s is referred to as the solution exchange time. Our estimates of channel opening rate are not significantly changed by assuming a sigmoidal time course for solution exchange (by squaring the right hand multiplicand of Eq. 3).[1] For all simulations, kinetic parameters were chosen so that the open channel probability was 0.5 at a fixed concentration of 20 μM ACh (Dilger and Brett, 1990). This allows us to compare predictions of onset time for parameters which produce essentially the same concentration-response relationship.

RESULTS

Currents elicited by rapid perfusion of millimolar concentrations of ACh arise from the opening of nearly all of the 50–100 nicotinic ACh receptor channels contained in outside-out patches from BC3H-1 cells (Dilger and Brett, 1990). Fig. 1 shows individual and averaged current records obtained from an outside-out patch held at +50 and −50 mV during application of 0.1 mM ACh at 11°C. The current rises to +120 or −150 pA as most of the ~100, 1.5 pA channels in the patch open. Desensitization occurs on the 100 ms time scale (Dilger et al., 1991) and is negligible here. The +50 mV averaged

[1]Other forms of the solution exchange profile can be imagined but would require additional parameters of some sort. One realistic idea is to consider receptors (normally?) distributed at different diffusional distances (times) from a source. However, simply increasing the solution exchange time for all receptors (as is done in our simulations) is a larger perturbation and requires only one parameter.

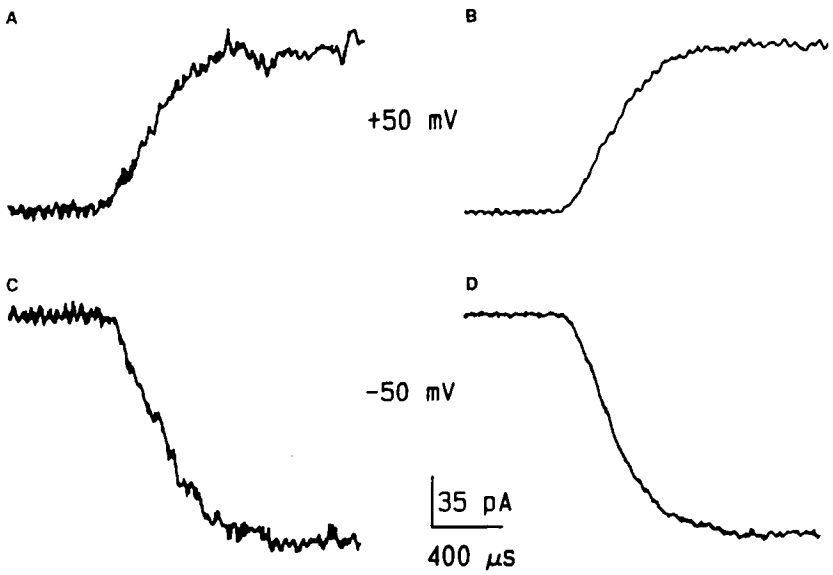


FIGURE 1 Examples of individual and averaged current records obtained during rapid perfusion of 0.1 mM ACh to a single outside-out patch from a BC3H-1 cell. (11°C, 10 kHz). (*A*) Individual current response obtained while the patch was held at +50 mV. (*B*) Ensemble averages of nine individual current responses at +50 mV. (*C*) Individual current response obtained while the patch was held at −50 mV. (*D*) Ensemble averages of nine individual current responses at −50 mV.

current trace is shown again in Fig. 2 along with averaged traces at 0.5, 2, and 10 mM ACh. There is a large decrease in onset time as the applied concentration is raised from 0.1 to 0.5 mM (430–140 μs). Thus, although 0.1 mM ACh nearly saturates the receptor binding sites (Dilger and Brett, 1990), binding is still the rate limiting process. In contrast, there is only a small decrease in onset time as the concentration is raised 20-fold beyond 0.5 mM.

Fig. 3 summarizes the results of experiments on seven patches. At both −50 and +50 mV, the onset time is a steep function of concentration between 0.1 and 0.5 mM ACh. At +50 mV, there is a relatively small decrease in onset time between 0.5 and 5 mM ACh (140–110 μs),

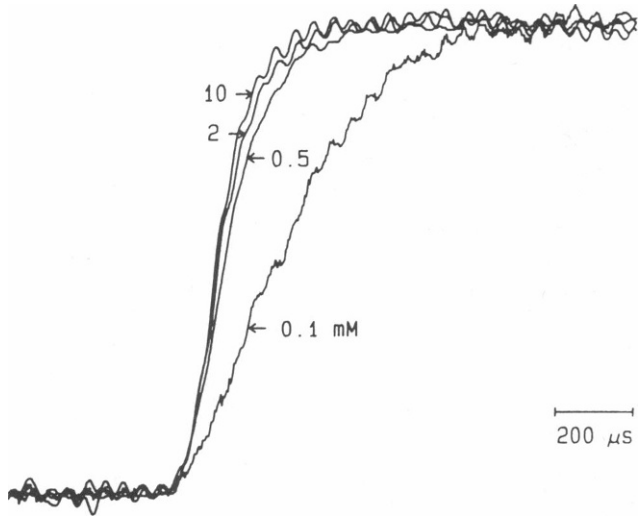


FIGURE 2 Averaged current responses from the same patch as in Fig. 1 to 0.1, 0.5, 2, and 10 mM ACh for an applied potential of +50 mV. Onset times (number of records averaged) were 360 (9), 150 (22), 135 (24), and 117 μs (37), respectively. Currents were scaled to the same peak value (peak currents are 115, 113, 96, and 88 pA, respectively). Filtered at 20 kHz except for 0.1 mM ACh at 10 kHz.

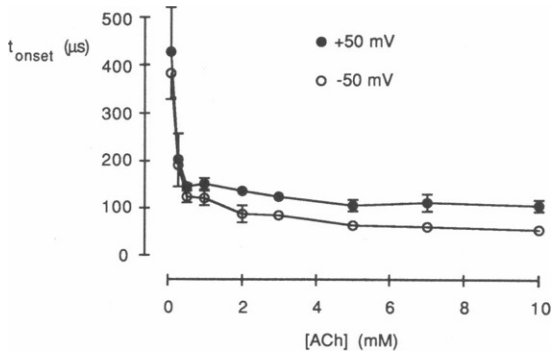


FIGURE 3 The acetylcholine concentration dependence of current onset time at +50 mV (*solid symbols*) and −50 mV (*open symbols*), 11°C. Each point corresponds to the average and standard deviation of onset times from 3–7 patches.

but the onset time is constant at 110 μs between 5 and 10 mM. At −50 mV, onset time is shorter for all concentrations of ACh and appears to reach a limiting value of 60 μs between 5 and 10 mM.

At millimolar concentrations of ACh, there is a fast, voltage-dependent block of open channels by free ACh ions (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985). At the level of single channels, the block manifests itself as a reduction in the apparent single channel current, which is intensified at negative potentials. In our experiments, block is seen as a reduction in ensemble mean current at concentrations >1 mM relative to 0.1 mM ACh. A knowledge of the kinetics of block is necessary for the interpretation of the results of onset time measurements at negative potentials (see Discussion). We assume that block by ACh is well described as a sequential block of open channels (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985) such that the equilibrium blocking constant, K_B, can be expressed as the ratio of unblocking and blocking rate constants k_{-b} and k_{+b}. K_B was determined from measurements of mean current amplitudes as a function of ACh concentration: K_B = 5.6 mM at −50 mV (Fig. 4; similar to that obtained from single channel analyses of BC3H-1 receptors at −50 mV, K_B = 10 mM, Sine and Steinbach, 1984) and $K_B \approx 200$ mM at +50 mV (data not shown). At the single channel level, block by ACh is so fast that filtering at 8 kHz obscures most of the brief gaps (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985) and variance (Ogden and Colquhoun, 1985) expected from a blocking process. The high signal-to-noise ratio realized in the "macroscopic" currents of our experiments allows us to filter at 30 kHz in our search for variance due to block. Comparison of the measured and expected variance is used to infer f_o, the half-power frequency of the blocking process (Ogden and Colquhoun, 1985). The results are summarized in Table 1. Even though the currents were filtered at 30 kHz, less than half of the noise due to block is detected. The average values of k_{+b} = 23/mM/ms (2.3 × 10^7/M/s) and k_{-b} = 130/ms are lower limits based on the assumption that all of the high-frequency noise is due to a single process. The agonist-induced noise power spectrum is flat up to the cut-off frequency of the Butterworth filter supporting our assumption that the measured variance arises from one or more high-frequency (≥30 kHz) processes.

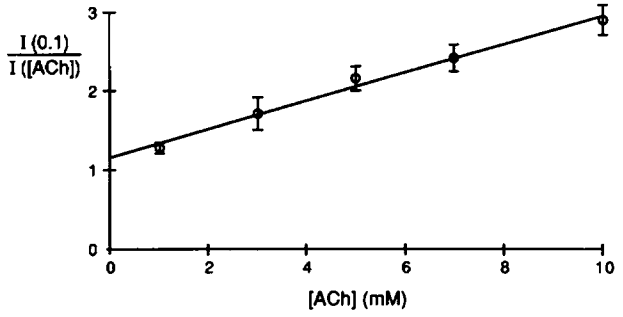


FIGURE 4 Channel block by ACh at −50 mV. The effect of block is to reduce the steady-state current during perfusion of millimolar concentrations of ACh. The graph illustrates the concentration dependence of the reciprocal of the steady-state current at blocking concentrations, I([ACh]), relative to the peak current at 0.1 mM ACh, I(0.1). Linear regression fit to Eq. 1 gave K_B = 5.6 ± 1 mM. Data represents the means ±SEM for 5–10 patches.

TABLE 1 **Results of excess channel noise analysis determination of blocking kinetics (5 patches)**

[ACh]	I	σ_m^2	σ_e^2	σ_m^2/σ_e^2	f_o	k_{-b}	k_{+b}
mM	*pA*	*pA²*	*pA²*		*kHz*	*ms⁻¹*	*ms⁻¹ mM⁻¹*
7	28.5	8.6	22.2	0.39	44	121	22
	85.1	22.6	66.4	0.34	48	134	24
	73.5	33.9	57.3	0.59	23	63	11
	232	45.8	181.	0.25	74	205	37
	40.1	13.1	31.3	0.42	39	109	19
Average	—	—	—	0.40	45	127	23
SD	—	—	—	0.12	18	52	9
10	29.7	7.6	27.2	0.28	63	142	25
	61.0	15.8	55.8	0.28	63	142	25
	68.1	25.8	62.3	0.41	39	88	16
	209.	38.7	191.	0.20	95	213	38
	32.9	13.6	30.1	0.45	35	78	14
Average	—	—	—	0.33	59	133	24
SD	—	—	—	0.10	24	54	10
All data	—	—	—	—	—	—	—
Average	—	—	—	—	—	130	23
SD	—	—	—	—	—	50	9

I is the "steady-state" (between 2 and 8 ms after onset) ensemble mean current elicited by rapid perfusion of blocking concentrations of ACh, σ_m^2 is the measured variance after subtraction of background, σ_e^2 is the expected variance, f_o is the half power frequency of block (estimated from the ratio of measured to expected variance using a graph of Eq. A1.10 of Ogden and Colquhoun, 1985), and k_{+b} and k_{-b} are the forward and reverse blocking rate constants. The equilibrium blocking concentration was taken to be 5.6 mM as determined from Fig. 4.

DISCUSSION

It is difficult to make a quantitative assessment of the speed of solution exchange achieved with a rapid perfusion system. The most commonly used method is to measure the current flow across the tip of an open patch electrode in response to a step change in electrolyte concentration. For our system, open electrode onset

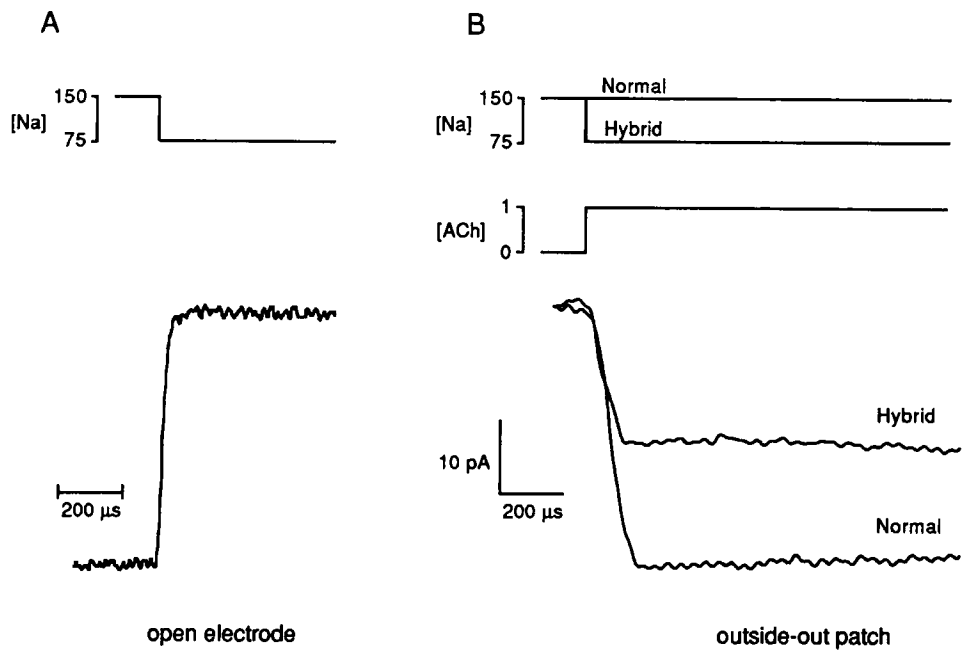


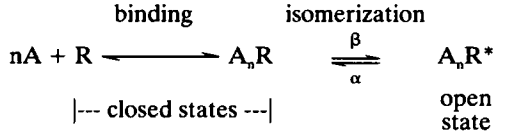
FIGURE 5 Two assessments of the speed of Na^+ exchange with the improved rapid perfusion system. (*A*) The current flowing through an open patch electrode during a change from normal extracellular solution to a 50% diluted solution. The current is driven by the change in electrode junction potential across the tip of the electrode. The 20–80% onset time of the current is 25 μs. Average of 15 records. (*B*) Onset responses from "normal" and "hybrid" solution exchanges across an outside-out patch containing ~30 ACh receptor channels. In the hybrid trace, the sodium ion concentration was decreased as agonist was added. Because the hybrid response is essentially a scaled-down version of the normal response, the reduction in Na^+ concentration around the patch must have been complete within 100 μs. (11°C, −50 mV, 1 mM ACh, 7 kHz). Average of 41 (normal) and 81 (hybrid) records.

times ranged from 15–35 μs but were typically 25 μs (Fig. 5*A*).[2] This method underestimates the time needed for concentration changes across an outside-out membrane patch attached to the electrode because the unstirred layer of solution surrounding the patch presents an additional diffusion barrier. The rapidity of ACh receptor desensitization prevents us from performing the identical experiment with Na^+ permeation through open channels in an outside-out patch. Instead, we made a *qualitative* assessment of the speed of Na^+ exchange across a patch by comparing the "normal" onset response (150 mM NaCl → 150 mM NaCl + 1 mM ACh, −50 mV) with a "hybrid" onset response in which we simultaneously applied agonist and reduced the NaCl concentration (150 mM NaCl → 75 mM NaCl + 1 mM ACh, −50 mV). The results are shown in Fig. 5*B*. In the normal response, the concentration of ACh around the patch when the current has leveled off may be only 20% of the applied 1 mM (0.2 mM ACh is more than sufficient to fully activate the receptors). If this were the case, the concentration of Na^+ would have reached only 135 mM at this point in the hybrid response. Thus, the hybrid response would exhibit a current decay from close to the normal steady-state amplitude to 50% of this amplitude as the $[Na^+]$ decreased relatively slowly to 75 mM. The absence of such a decay suggests that the $[Na^+]$ at this patch reached 75 mM within 100 μs (a conservative estimate). Hybrid currents from some patches did show a decay; further evidence that solution exchange times vary from patch to patch. We would like to measure the time needed to perfuse a patch with ACh (especially because the diffusion coefficient of ACh is 2.5 times smaller than that of Na^+) but any such measurement is likely to be confounded by the kinetic actions of ACh on the receptor-ion channel.

Ideally, the only assumption necessary to interpret the

[2]Using a similar system, Maconochie and Knight (1989) achieved solution exchange across an open electrode in 200 μs. The faster response of our system (25 μs) may be due to a shorter distance between the electrode and the perfusion tubes (< 1 mm in our system, 7 mm in theirs) allowing less time for diffusion or mixing across the solution interface before it reaches the electrode. Their system may be slower with excised patches as well. Perfusion of 0.1 mM ACh at room temperature resulted in an onset time of < 200 μs in our system and 2 ms in theirs. However, the possibility of differences in the kinetics of ACh receptor channels from BC3H-1 cells and C2 cells precludes a direct comparison of the solution exchange performance of the two systems on excised patches.

results of onset experiments would be that activation of channels takes place in two, sequential steps: the binding of n molecules of ACh (A) to the receptor (R) followed by a conformational change (isomerization) corresponding to the opening of the channel's gate (Scheme I).



SCHEME I

In this scheme, the onset of current upon rapid perfusion of ACh, depends upon both ACh binding and channel isomerization. The rate of binding is given by the product of the bimolecular binding rate constant and the concentration of ACh. By increasing the concentration of ACh applied, it should be possible to increase the speed of the binding steps such that channel opening becomes rate limiting; further increases in concentration would not result in a faster onset. In the limiting case, onset time is a function of only the opening (β) and closing (α) rates of the channel,

$$t_{onset} = 1.39/(\alpha + \beta). \quad (4)$$

Because the opening rate exceeds the closing rate,[3] a measure of the limiting value of t_{onset} provides a first approximation to the opening rate itself.

For experiments at +50 mV, it appears that ideal conditions have been realized. The presence of the plateau between 5 and 10 mM ACh in Fig. 3 suggests that the concentration change occurs sufficiently fast so that the concentration-independent step, channel opening, has become rate limiting. Thus, t_{onset} = 110 μs and β ≈ 12/ms. It would be more satisfactory, though, if an estimate of the solution exchange time were available. A qualitative estimate is provided by studying some of the onset currents obtained under conditions where block by ACh is significant. Examples from two different patches held at −50 mV and perfused with 3 and 7 mM ACh are shown in Fig. 6. In both of the 7 mM examples, the onset of the current is rapid (50–60 μs), but the trace in Fig. 6 *A* has a decay phase not seen in that of Fig. 6 *B*. This decay, which is never seen at +50 mV in this concentration range (although it is seen for applications of ≥ 30 mM ACh), arises from progressively more severe

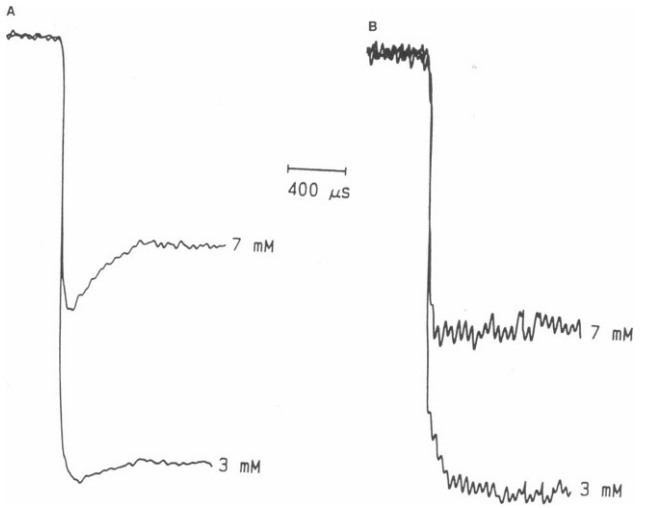


FIGURE 6 Current onset traces at −50 mV applied potential for two different patches; one having a relatively slow solution exchange (*A*) and the other a very fast solution exchange (*B*). Onset currents resulting from rapid perfusion of 3 and 7 mM ACh are shown. The "steady-state" current is smaller in the 7 mM traces because of channel block by ACh. A slower solution exchange results in the overshoot and decay of the current in (*A*). The two patches contained different numbers of channels; the peak currents at 3 mM ACh were 262 pA (*A*) and 64 pA (*B*). Ensemble averages of 47–50 records except for 7 mM trace in (*A*) which is 10 records.

channel block by ACh as the concentration increases relatively slowly from >3 to 7 mM ACh. Because the trace in Fig. 6 *B* does not exhibit this decay, the concentration change was faster for this patch. Onset times measured at +50 mV and 7 mM ACh were similar for both the "slow" and "fast" patch. We conclude that the speed of the solution exchange is adequate to validate Scheme I for the interpretation of onset results at positive potentials.

The presence of block makes it unlikely that a similar analysis would be valid for the −50 mV results. The combination of fast block and a finite solution exchange time distorts the current onset, onset becomes faster under these conditions. Consider two onset responses obtained from a noninstantaneous solution exchange such that fast block was "switched on" for one response only. At early times, the currents superimpose because the agonist concentration is below the blocking range. However, the blocked current levels off sooner than the unblocked current because its steady-state amplitude is smaller. Thus, the 20–80% onset time is shorter for the blocked current. One of two conditions would have to be met for onset time to be a reliable indicator of channel isomerization when block is present: (*a*) block is slow (relative to isomerization) or (*b*) solution exchange is fast (relative to block and/or isomerization).

To determine channel opening rate at negative poten-

[3]At −50 mV, the maximum open channel probability exceeds 0.9 (Dilger and Brett, 1990; Dilger et al., 1991). At +50 mV, direct measurement of open channel probability in BC3H-1 cells has not been made, but we find that it is ~0.8. The mean channel open time at +50 mV is ≤ 1 ms (Dilger et al., 1991), giving an estimated closing rate of 1000/s.

tials, it is necessary to assume a specific kinetic reaction scheme. Scheme II represents the best supported kinetic model for activation and block of the ACh receptor (Sine and Steinbach, 1984, 1986, 1987; Colquhoun and Sakmann, 1985; Ogden and Colquhoun, 1985; Colquhoun and Ogden, 1988; Jackson, 1988; Sine et al., 1990), sequential binding of two agonist molecules for channel activation, isomerization, and sequential block of open channels.

$$R \underset{k_{-1}}{\overset{2k_{+1}}{\rightleftharpoons}} AR \underset{2k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^* \underset{k_{-b}}{\overset{k_{+b}}{\rightleftharpoons}} A_2R^*A$$

|-------- closed states --------| open state | blocked state

SCHEME II

Numerical simulations of Scheme II were performed for different values of the kinetic rate constants and solution exchange time, τ_s. Comparison of simulated and experimental responses was made by examining both the onset time and decay amplitude, the ratio of peak to steady-state current (see Fig. 6).

A lower limit on β can be obtained from the onset time at 0.5 mM ACh; a concentration for which activation is complete, but block is not yet significant. Because t_{onset} = 115 μs at this concentration, β must be at least 12/ms. Further information about β comes from simulation. For concentrations >5 mM ACh, the parameters which determine onset time and current decay amplitude are β, τ_s, and the blocking rate constants k_{+b} and k_{-b}. Because we determined the blocking rate constants independently, we systematically varied the remaining two factors in the simulation program. These results are shown in Fig. 7, *A* and *B*, for simulations at 7 and 10 mM ACh, respectively. The predictions for onset time (ordinate) and decay amplitude (abscissa) are plotted as contour lines whereas the experimental values obtained in different patches are denoted by symbols. Estimates of β and τ_s are obtained from interpolation between simulation contours. Solution exchange times range from 100–400 μs in different patches but the channel opening rate is consistently 10–17/ms.

We tested the generality of this result by varying the rate constants used in the simulation. For 7 and 10 mM ACh, $\beta \approx 15$/ms for a large range in cooperativity of binding ($0.01 \leq K_1/K_2 \leq 100$).[4] However, it is unlikely that there is extreme positive or negative cooperativity for binding of ACh in BC3H-1 cells because simulations using $K_1/K_2 > 10$ and $K_1/K_2 < 0.1$ gave predictions of onset time at 0.1 mM ACh considerably longer than our

[4]This range includes estimates of cooperativity of ACh binding derived from experiments (Sine and Steinbach, 1987; Colquhoun and Ogden, 1988; Sine et al., 1990) and theoretical considerations (Jackson, 1989).

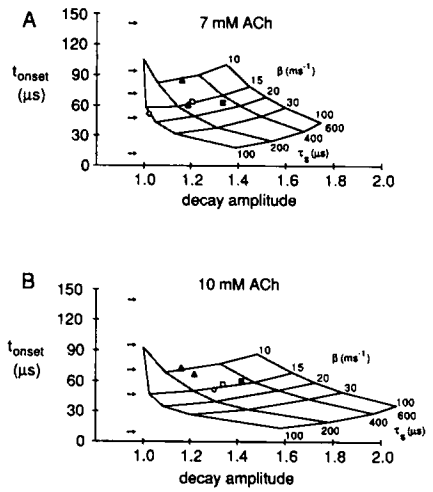


FIGURE 7 The results of numerical simulation of Scheme II for various values of channel opening rate, β and solution exchange time, τ_s. For each of the 20 combinations of β and τ_s, onset time (ordinate) and decay amplitude (abscissa) are plotted as the intersections of contour lines. The experimental data from five patches are represented by different symbols. (*A*) 7 mM ACh, (*B*) 10 mM ACh, −50 mV. Arrows indicate the onset times which would be achieved by an instantaneous solution exchange. Values for the microscopic rate constants of Scheme II: $k_{+1} = k_{+2} = 10^8$/M/s, β as indicated, α = 300/s, $k_{+b} = 2.3 \times 10^7$/M/s, $k_{-b} = 1.3 \times 10^5$/s. k_{-1} and k_{-2} were determined for each value of β such that the open channel probability was 0.5 at 20 μM ACh ($k_{-1} = k_{-2}$ = 9.6, 12.2, 14.3, 18.0, and 34.5 × 10^3/s for β = 10, 15, 20, 30, and 100 × 10^3/s, respectively).

experimentally observed values. Increasing or decreasing the blocking rate constants by a factor of two (while holding K_B constant) had no significant effect at any concentration. Changes in K_B, however, did influence the estimation of β. The uncertainty of ±1 mM in K_B translated into an uncertainty of ±2/ms in β.

Unlike the channel closing rate, the opening rate is relatively insensitive to voltage. The closing rate of receptors from BC3H-1 cells has been found to increase *e*-fold per 100–130 mV depolarization (Sine and Steinbach, 1986; Dilger et al., 1991). The results shown here indicate that a depolarization of 100 mV decreases the opening rate by only 25%.

A complicating feature of nicotinic ACh receptors is the existence of more than one component in the open time distribution of single channels. In some preparations, a brief open component persists even at high agonist concentrations (Colquhoun and Sakmann, 1985; Sine and Steinbach, 1987). Its association with the states in Scheme II is not known, but it does not appear to originate from a separate population of ACh receptor channels (Sine and Steinbach, 1986). In rapid perfusion experiments, there are two components in the decay of current after *removal* of agonist from the patch; the

faster component accounts for 20–30% of the total current and is probably analogous to the brief open time component (Liu, Y. and J. P. Dilger, manuscript in preparation). Therefore, onset currents do not arise from activation of a single open state of the channel. Onset currents, though, exhibit only one component implying that either both components activate simultaneously or the brief open state activates too quickly to be resolved. In either case, because the receptors are fully activated within 100 μs, the onset we measure must correspond to the long-lived open state (if not both states), the better characterized state in single channel experiments.

We performed several onset experiments at 22°C to estimate the temperature dependence of channel opening rate. At +50 mV, onset times were 55–70 μs for 5–10 mM ACh, 1.5–2.0 times faster than the corresponding onset times at 11°C. This translates into a temperature sensitivity of channel opening rate which is greater than that which could be explained by diffusion alone and must arise from conformational changes within the ACh receptor protein. Similarly, Dwyer (1981) calculated a Q_{10} of 2.3 for the rising phase of miniature endplate currents.

Estimates of channel opening rate of ACh receptor channels determined by various methods are shown in Table 2. Receptors from different preparations may indeed have different intrinsic opening rates, but the method used may play a more significant role. In principle, noise analysis (Sakmann and Adams, 1978) furnishes essentially the same information as the experiments described here. In practice, though desensitization limits the usefulness of noise measurements to concentrations below 0.1 mM ACh, concentrations for which onset time is more sensitive to agonist binding rate constants than to the channel opening rate. Interpretation of end-plate current onset measurements relies heavily on models of synaptic events (Land et al., 1980, 1981, 1984; Dwyer, 1981). It was anticipated that single channel measurements would provide the ultimate determination of opening rate but hopes were tempered when the complexity of channel kinetics on the submillisecond time scale was realized (Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986, 1987). At least two brief components are seen in closed time histograms, and it is difficult to decide which one of these gaps arises from channel isomerization. Our results indicate that the shorter gap component seen in channels from BC3H-1 cells (45 μs instead of 600 μs, Sine and Steinbach, 1986) is the one which corresponds to isomerization.

Because the opening rate is so rapid, we might question whether our onset experiments provide a lower limit for β rather than β itself. This is particularly apposite because in single channel kinetic experiments, the opening rate is comparable to or faster than the temporal resolution of the recording system and its value must be determined by extrapolation. Our simulations for −50 mV predict that when β is considerably larger than our estimate of 15/ms, decay amplitudes >1.4 should be apparent in onset currents at 7 and 10 mM ACh (Fig. 7, contour for β = 100/ms). Because these decays become increasingly more pronounced for slower solution exchange times, our ability to detect them is not constrained by experimental limitations. Similarly, at +50 mV, increasing B to 100/ms would result in an easily detectable 30% decrease in onset time between 5 and 10 mM ACh for solution exchange times >400 μs. We conclude that unless the present models of ligand-gated channel activation are inadequate, onset time measurements furnish a reliable estimate of the channel opening rate.

The rapid perfusion method described here may be useful in the determination of opening rate for other ligand-gated ion channels. It is not necessary for the solution exchange to be faster than channel activation, only that high enough agonist concentrations be used so that binding is not rate limiting. At 10 mM ACh, for example, even when the solution exchange time is 400 μs, saturating concentrations of ACh (several hundred micromolar) reach the patch within tens of microseconds and binding prolongs the 100 μs isomerization time by only 10%. Low affinity actions of agonists (such as

TABLE 2 A comparison of the opening time of ACh receptor ion channels by ACh as determined by different techniques

Method	1/β	T	V	Preparation	Reference
	μs	*°C*	*mV*		
Noise analysis	500	10	−80	frog muscle	Sakmann and Adams, 1978
MEPC onset	55–80	22	−100	lizard muscle	Land et al., 1981, 1984
Single channels	20	11	−140	frog muscle	Colquhoun and Sakmann, 1985
Single channels	70 or 3000	11	−100	BC3H-1 cells	Sine and Steinbach, 1986
Rapid perfusion	70	11	±50	BC3H-1 cells	present paper

The opening time is defined as the reciprocal of opening rate, β. (MEPC—miniature end plate current)

block and desensitization) may limit the maximum concentration which can be used. We managed to avoid these complications at positive potentials and as a result, our measurement of the opening rate of nicotinic ACh receptor channels at +50 mV is both direct and independent of most assumptions about the details of the molecular processes which lead to channel opening.

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