Block by Acetylcholine of Mouse Muscle Nicotinic Receptors, Stably Expressed in Fibroblasts

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ABSTRACT We have measured the concentration and voltage dependence of block by acetylcholine (ACh) of fetal- and adult-type mouse muscle nicotinic receptors, expressed in a fibroblast cell line. Data, obtained at a transmembrane potential of ~60 mV and with ACh concentrations of 1 mM and above, are broadly consistent with the occlusion of an open channel with a single ACh+ ion (simple open channel block). The rate of recovery from block is ~40,000 s⁻¹ and has only a weak voltage dependence. This is in contrast to the strong voltage dependence observed for the degree of block. Deviations from the predictions of the simple model are seen in data collected at positive transmembrane potentials and at negative potentials for ACh concentrations <1 mM. Less concentration dependence is observed than expected. Of a number of models tested, we demonstrate that two models incorporating both a high and a low affinity blocking site can predict our data.

INTRODUCTION

It has been known for some time that ACh is an agonist and an antagonist for the nicotinic acetylcholine receptor (AChR), both activating the membrane current and, at high concentrations, reducing or inhibiting the current. Two distinct types of inhibition of the ACh-dependent current by high ACh⁺ concentrations have been described: a slow but reversible loss of responsiveness or "desensitization" to ACh (Thesleff, 1955; Katz and Thesleff, 1957) and a rapid "channel block" (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985).

Desensitization is thought to reflect a slow conformational change resulting in a liganded but inactive state. Desensitization appears to be promoted by the same processes that result in activation (opening of the channel) of the receptor and is generally thought not to require the binding of additional ACh⁺ ions. The desensitized receptor has been shown to have a higher affinity for ACh (for review see Ochoa, Chattopadhyay, and McNamee, 1989), as predicted by Katz and Thesleff (1957).
Inhibition also can develop and dissipate very rapidly with high ACh concentrations. This rapid self antagonism has most commonly been ascribed to a mechanism in which an ACh ion directly occludes the ion channel pore (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985). This is frequently described using a simple linear kinetic model that includes "open channel block." It is usually assumed that while the channel is blocked, no other reactions may take place. Other such disparate agents as procaine (Fatt and Katz, 1951; Adams, 1977), barbiturates (Adams, 1976), and chlorisondamine (Neely and Lingle, 1986) are also thought to exert their antagonism at the nicotinic receptor by this mechanism. On the whole, data have been generally in accordance with this model. As originally proposed, recovery from block requires unblocking to an open channel. Most deviations from this model involve the assumption that blocked channels can isomerize in some way to states comparable to a liganded closed state ("trapping" block) (Gurney and Rang, 1984; Neely and Lingle, 1986) or a desensitized state (Macnochie and Knight, 1992b), or follow some other unspecified pathway, bypassing the open state, to the inactive unliganded form (Neher, 1983; Steinbach, 1968). However, even when observed deviations from the simple model are taken into account, it is still generally assumed that occupancy of the blocking site by a blocking particle is sufficient to prevent the passage of a current through the channel.

That open channel block involves a binding site for the blocking agent within the channel pore (and partway across the transmembrane electric field) is prompted by the observed voltage dependence of block. Using the rate theory of Eyring and Eyring (1963), Woodhull (1973) formalized a single-site, symmetrical barrier model of open-channel block and showed that it accounted well for the voltage dependence of the block of sodium channels by protons. The binding site was postulated to be partway through the channel, so that a blocking ion interacts with the transmembrane electric field upon entering and leaving the binding site. As would be expected, therefore, the block of nicotinic channels by uncharged noncompetitive inhibitors such as benzocaine (Ogden, Siegelbaum, and Colquhoun, 1981) or barbiturates (Adams, 1976) has a low voltage sensitivity. The blocking effect of charged or polar drugs, on the other hand, has a voltage dependence that has been described in terms of Woodhull's approach (Adams, 1977; Neher and Steinbach, 1978). However, when block is analyzed over a sufficiently wide voltage range, it becomes clear that the simple analysis is inadequate, as the charge on the blocking drug must be assumed to sense more than the total applied field (Sine and Steinbach, 1984; Carter and Oswald, 1993). Hille and Schwarz (1978) modeled the block of potassium channels by monovalent cations and found that a multi-ion pore model can account for the high degree of voltage sensitivity observed. As an alternative, Sine and Steinbach (1984) point out that the major features of the Woodhull model can be retained if some voltage-sensitive change in the channel structure can lead to an alteration of the barrier heights.

The placement of the blocker-binding site partway through the channel and thus partway across the transmembrane field is not the only way to confer voltage sensitivity to noncompetitive inhibition. A model involving separate binding and isomerization steps is also consistent with most data on channel block (Neher and Steinbach, 1978; Sine and Steinbach, 1984). A good fit may be obtained with the voltage sensitivity confined to the isomerization reaction and incorporating terms...
in both $E$ and $E^2$ (Sine and Steinbach, 1984). However, a major objection to such a model is that, as we have noted, block by uncharged agents shows no or little voltage sensitivity (Ogden et al., 1981; Adams, 1976).

Using fast solution changes to apply high concentrations of ACh to outside-out membrane patches taken from cells expressing fetal- or adult-type muscle AChR subunits, we have investigated block further and tested our data against some of these models.

**METHODS**

**Cell Culture**

Quail QT-6 fibroblasts were transfected with cDNAs coding for muscle AChR subunits and for resistance to the antibiotic geneticin. Stable clones were selected as described (Phillips, Kopta, Blount, Gardner, Steinbach, and Merlie, 1994; Kopta and Steinbach, 1994). Clones expressing subunits for the fetal-type muscle AChR (Q-F18 cells; α, β, δ, and γ subunits) and adult-type AChR (Q-A33 cells; α, β, δ, and ε subunits) were used for the present studies. Cells were grown in medium 199 (Gibco Laboratories, Grand Island, NY) containing 10% tryptose phosphate broth (Gibco Laboratories), 5% fetal bovine serum (HyClone Laboratories, Logan, UT), 1% dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO), 100 U ml$^{-1}$ penicillin, 100 μg ml$^{-1}$ streptomycin, and 150 μg ml$^{-1}$ G-418 (Gibco Laboratories).

Populations of cells expressing a high density of surface AChRs were obtained by selective adhesion (Barker, Worman, and Smith, 1975) to the surface of plastic Petri dishes coated with monoclonal antibody mAb-35 (Chen et al., 1995). mAb-35 binds to an external epitope on the α subunit of the AChR (Tzartos, Rand, Einarson, and Lindstrom, 1981) and was purified from the supernatant of cultures of monoclonal antibody hybridoma cells (American Type Culture Collection, Gaithersburg, MD) by ammonium sulfate precipitation and hydroxyapatite chromatography (Harlow and Lane, 1988). Cells were passaged every 3–4 d by dissociating them with trypsin solution (0.05% trypsin in a saline containing [in millimolar]: 139 NaCl, 5.4 KCl, 5.6 glucose, and 0.5 EDTA [ethylenediaminetetraacetic acid]). Cells for electrophysiology were plated on collagen-coated glass coverslips in growth medium without G-418 and used for electrophysiology between 24 and 48 h.

**Recordings**

Outside-out patches were obtained using standard techniques (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). Recording pipettes of 3–5-MΩ resistance were fabricated from Kimax (Kimble Glass, Toledo, OH) standard wall glass (1.4-mm OD) and were fire polished. Series resistances in the whole-cell mode were about twice the resistance of the open pipette tip. All currents were recorded using an EPC7 amplifier (List, Darmstadt, Germany) in the low gain range at full bandwidth (100 kHz, −3 dB) and were acquired directly to a hard disk at 0.5 or 1 MHz (DAS50 Keithley-Metrabyte, Taunton, MA). All experiments were performed at room temperature (20–23°C).

Extracellular solution contained (in millimolar): 150 NaCl, 5 KCl, 2 MgCl$_2$, 2 CaCl$_2$, and 10 HEPES (N-[2-hydroxyethyl]piperazine-N'-(2-ethanesulfonic acid)), (pH 7.4 with NaOH). Pipette filling solution for outside-out patch recordings contained (in millimolar): 140 CsCl, 2 MgCl$_2$, 1 EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid), 0.1 CaCl$_2$, 10 HEPES, 10 tetraethylammonium, 5 Na$_2$ATP, and 0.3 dithiothreitol (pH 7.4 with NaOH).

**Solution Changes**

Solution changes were made essentially according to the methods of Maconochie and Knight (1989), except that faster perfusion speeds were used, enabling faster jumps to be applied. The
Valve switching, data acquisition, averaging, and display were run from routines written in Assembler as part of OLAV, a DOS-based custom application.

Although the solenoid valves used to switch solutions were driven by logic pulses that were timed to an accuracy of 1 \( \mu \)s, the exact moment of the solution change varied a little from one application to another. We attribute this to chaotic fluid motions in the recording bath. The best re-

![Figure 1](https://example.com/figure1.png)

**Figure 1.** The time course of solution change. Trace A is the average of 37 responses of a leaky outside-out patch to a jump in the concentration of external NaCl from 150 to 300 mM. Trace B is the average of 42 responses of the open pipette to the same stimulus, after the patch had been blown away. Traces A and B show the abrupt changes in junction current upon changing the ion content of the external solution. Trace C is the average of 11 responses of the patch to a simultaneous application of 100 \( \mu \)M Ach and the equi-osmolar replacement of 30\% of the external NaCl with mannitol. The current in this case is due to the opening of nicotinic receptors on the patch. The current suddenly increases at the end of the application, because the single-channel conductance is abruptly increased when the external NaCl concentration is restored. Trace D is the average of 11 responses of the same patch to 10 mM Ach alone and is the normal response of a Q-F18 cell membrane patch to a blocking concentration of Ach. The sudden increase in the current at the end of the application in this case is due to channels that are active and blocked suddenly unblocking. Traces A, C, and D were recorded from the same patch, and trace B is from the same pipette. Traces B, C, and D are displayed at the same scale. E shows part of traces A–D overlaid. The section chosen is the end of the stimulus. Each trace has been scaled (and inverted where necessary) and aligned so that the end of each stimulus is coincident. The open symbols are from trace A, the thin curve is from trace B, the closed symbols are from trace C, and the thick line is from trace D. This figure shows that the three methods designed to estimate the time course of the solution change produce comparable results, whereas the time course of the unblocking of the nicotinic receptors is slower. All traces were recorded at the bandwidth of the EPC7 in the low gain range, digitized at 1 MHz, and digitally filtered for display at 50 kHz (sinc).
results are obtained if the depth of the bath is kept constant. Then the moment of the solution change is reproducible to \( \sim 10 \mu s \).

We measured the speed of the solution change in three ways. The first was the standard approach of changing the external NaCl concentration at the tip of an open patch pipette (Fig. 1 B). Before the start of an experiment and several times during the day, the solution change was checked by doubling the external NaCl concentration at an open patch pipette. Data were not recorded unless the 10–90% rise time of the junction current was 30 \( \mu s \) or less (20 \( \mu s \) was typical). In many instances, to examine the effect of the presence of a patch at the pipette tip, this estimate was checked by following the junction current on doubling the external NaCl concentration at a

![Figure 2](image)

**FIGURE 2.** The alignment of individual responses. This figure shows how successive data traces were aligned for averaging. The traces displayed here were recorded from an outside-out patch taken from a Q-F18 cell (the average is shown in Fig. 1 A) and shown the current upon the restoration of the normal NaCl concentration at the end of a test pulse. The solid line represents a single response of this leaky patch to a change in external NaCl concentration (filtered at 10 kHz; sinc); the circles follow the average of 37 single responses (filtered at 50 kHz; sinc). Although the single response is shown filtered, most of the calculations described here were performed on unmodified data. A step is known to have occurred in the region between the two arrows. An initial estimate is made of the position of the step by examining the early components of the Fourier transform of this region. There is a constant phase difference between consecutive components, which is most obvious in the early components (e.g., the leftmost four points in the inset, which is a plot of the phase of the first 40 components of the Fourier transform of the data between the two arrows). The size of the phase difference is related to the location of the step and may be calculated from a straight line fit (wrapped around \( + \) or \( - \pi \)) to the first few phase components (see lines drawn on the inset). Next, two sections of this response were chosen in fixed relation to the initial estimate (outlined by the boxes). From these, averages of the current before and after the solution change were made. A threshold was set at some fixed fraction (typically 0.5) of the difference between the current before and after the solution change. Traces were then aligned by filtering the record (see Methods) and using the first point in the filtered trace that crossed the threshold as the index point for aligning the unfiltered record.

leaky outside-out membrane patch (Fig. 1 A). The results obtained with each method are similar. As an additional check, current jumps were induced in open nicotinic channels by the application to an outside-out patch of 100 \( \mu M \) ACh in a low cation solution (an extracellular solution that was 30% replaced with an equiosmolar mannitol solution). Upon removal of the ACh-containing solution, there was an immediate increase in the current (Fig. 1 C) due to the restoration of the single-channel conductance. The rise time of this current was complicated by the normal closure of nicotinic channels upon removal of ACh, and the time course was a little slower than that obtained from the other two methods, but it was faster than a typical data trace (see Fig. 1 E).
Data were digitally filtered, aligned, averaged, fitted with sums of exponentials, and plotted using a PC clone and the software package VIEWMENUS (available as DELPX02 from the following world wide web sources: gopher://sunsite.doc.ic.ac.uk/1/computing/systems/ibmpc/simtel/msdos/math http://www.acs.oakland.edu/oak/SimFel/msdos/math.html).

Alignment of data. The elements of the alignment process are shown in Fig. 2. The alignment procedure is essentially a threshold crossing method. To make the procedure independent of variations in current baseline and equivalent for events of different sizes, the threshold was determined for each trace separately, from estimates of the current just before and just after the event. This required an initial estimate of the position of the event, which was made by an examination of the Fourier representation of part of the data trace.

Data are in the form of consecutive single responses, in which for one reason or another, the event to be aligned is misplaced (typically by 10–100 μs). Cursors were placed ~1 ms to either side of the start of the event. To obtain an initial estimate of the position of the event, a Fourier transform of the data section between the two cursors was taken, ff the data consist of a step function plus noise, the phases of the low order Fourier components form an arithmetic progression whose difference is determined by the position of the step. If the data are symmetrical, this difference is 0. So by finding the phase difference of the first two to four Fourier components, an estimate of the position of the step in relation to the two cursors can be obtained. Next we took means of data sections before and after the event and set a threshold that was some fraction of the difference between the two means. Finally, consecutive events were aligned on the first point past the threshold. To avoid alignment on spurious threshold crossings due to noise, the alignment point was determined from data filtered at a frequency at which the rising phase had no turning points. After the determination of the alignment point, we averaged unfiltered data. With most data, the high signal-to-noise ratio meant that the choice of the filter was not critical.

Digital filtering. Filtering of data was performed after acquisition by convolution with an appropriate impulse response function. For most purposes a sinc ($\sin x / x$) function was chosen, truncated at the third minimum or above by a raised cosine window.

Fitting with sums of exponentials. Aligned and averaged data were fitted with sums of exponentials using the differential equation, Legendre polynomial (DELP) method of Martin, Maconochie, and Knight (1994). This method involves the linear least-squares fitting of the coefficients of an auxiliary equation to the data. If the behavior of the receptor is modeled by a first-order kinetic scheme and the transition rates in the scheme are incorporated in a Q matrix (Colquhoun and Hawkes, 1977), then the characteristic equation of the Q matrix is the one whose coefficients are determined by DELP. The exponential rate constants are the roots of the characteristic equation. The amplitudes of the exponential components are found by a further linear least-squares fitting routine.

Estimating the ratio of blocked to unblocked channels. The simplest approach to measuring the ratio of blocked to unblocked channels is to measure the current just before ($I_b$) and just after ($I_b + I_u$) removal of ACh from the patch. If the recovery of current from the blocked state were instantaneous, then this would be the correct approach. However, blocked channels take some time to unblock, and in the process, some channels will close, leading to an underestimate of the amount of block. This is particularly apparent at positive potentials, at which only a small part of the current is blocked. By examining artificially generated data, we determined that by using the peak of the unblocking transient we underestimate the true blocked fraction by ~10% when a sizeable amount of block is anticipated (10 mM, −60 mV) and by a factor of ~3 for small amounts of block (1 mM, +40 mV).

An alternative is to back-extrapolate the falling phase of the current to the point at which ACh was removed from the patch. Again, modeling shows that this leads to an overestimate of the
amount of block by $\sim 10\%$ when much block is expected and by a factor of 1.5 when little block is expected. Under the circumstances, the latter method is to be preferred, although the true amount of block lies between the two estimates. In practice, we fitted that part of the current after removal of ACh with a sum of two to four exponentials. The first component describes the increase in current as channels unblock, and the sum of the remaining amplitudes is taken to be the current $I_0 + I_B$. So if the current after the ACh is removed from the patch is fit with

$$A_n e^{-t} + A_{n-1} e^{-t} + \ldots + A_0,$$

then $\lambda_n$ is the unblocking rate, and

$$I_0 + I_B = \sum_{i=0}^{n-1} A_i.$$

**Series resistance error correction.** With a few data records, currents were large enough that the series resistance voltage error was greater than a few millivolts. In these cases, two approaches were taken. For data giving the concentration dependence of block, a purely ohmic correction was made to the digitized record given the holding potential and the series resistance. For data related to the voltage dependence of block, for which an accurate knowledge of the transmembrane potential is more important, the transmembrane potential was calculated from the series resistance and the current in the presence of ACh. The value of the current $I_0 + I_B$ after the removal of ACh was adjusted to the value that would be expected if the transmembrane potential were the value calculated from the current before removal of ACh.

Although the EPC7 patch-clamp amplifier has a mechanism for electronically reducing the effective series resistance by means of a positive feedback circuit, in practice this can be done only if the bandwidth is restricted. Moreover, the signal-to-noise ratio is degraded. For these reasons, we never used this function.

**A smoothed representation of a real solution change profile.** We started with a set of junction currents measured at an open patch pipette in response to jumps in the external NaCl concentration. These were aligned as previously described and averaged. Four sections of the average record were fitted with a single exponential. The fitted functions were strung together to make a continuous record. The four sections were as follows: from the start of the data record to the point at which the junction current has changed by 50%, from this halfway point to the middle of the application, from somewhere in the middle of the application to a point where the current is 50% of the way back to its resting value, and then from there to the end of the record. Any offset was subtracted so that the baseline value was 0, and the record was normalized so that the maximum value was unity. To simulate the agonist concentration as a function of time, it was then necessary simply to multiply this normalized record by the concentration required.

**Synthesized solution change profiles.** Sigmoidal shaped profiles were generated from the following function:

$$G = \begin{cases} 0.5 e^{1/\tau} & t < 0 \\ 1 - 0.5 e^{-1/\tau} & t > 0 \end{cases}$$

**Predicting the voltage dependence of the high concentration cluster mean current.** Sine and Steinbach (1984; Fig. 2) showed the voltage dependence of the mean current during clusters of single-channel openings for several agonist concentrations. To display the concentration dependence of the Sine and Steinbach data at +40 and -60 mV, we averaged individual measurements of the current amplitude at the nominal potentials ($\pm 10$ mV).

For each model, the predicted occupancy of the open states as a function of both concentration and potential was multiplied by the expected single-channel conductance for each potential. This was estimated from the single-channel conductances obtained in 500 nM ACh (Fig. 11, column i,
A smoothed representation of the expected single-channel conductance was obtained by fitting with an arbitrary function of potential alone and the function used to obtain values of the conductance at discrete potentials. With each model, reaction rates and voltage sensitivities were varied until the best fit by eye was obtained to both the voltage dependence and the concentration dependence of the Sine and Steinbach (1984) data (Fig. 11, columns i and ii).

Generating ensemble current predictions. For each model, the rate constants that best predict the data of Sine and Steinbach (1984) were used to generate the time dependence of each state of the model. The time dependence of the open states was summed to predict the ensemble current.

For the most part, the eigenvalue approach (Eigen and de Maeyer, 1963; Colquhoun and Hawkes, 1977) was used. Subroutines hqr and Invlt (Press, Teukolsky, Vetterling, and Flannery, 1992) were used to calculate the eigenvalues and eigenvectors, respectively, of the model reaction scheme. In a few cases, in which failure of the eigenvalue approach was speculated, we checked the results using either a Monte-Carlo or Runge-Kutta method (subroutine Odeint, with adaptive step-size control; Press et al., 1992). Our implementation of the eigenvalue and Runge-Kutta approaches is the program tstre/ax, written in Pascal.
RESULTS

Fig. 3 displays most of the essential features of this study. Fig 3 A shows the currents recorded from a single outside-out patch taken from a Q-A33 cell in response to very short applications of 1–10 mM ACh and at two transmembrane potentials: +40 and −60 mV. With this patch, the currents did not appear to "run down" (irreversibly lose the response to ACh) quickly with time, making a qualitative comparison of responses to different concentrations easier. Responses to a wide range of ACh concentrations were possible, and the size of the currents was such that we could see even the small amount of block present at +40 mV in response to 1 mM ACh. With other patches, currents were not always as large, they often ran down over the course of an experiment, and data could not always be obtained over both a wide concentration range and at two potentials. Nevertheless, we could still obtain useful measurements of block.

The peak current during an application of a high concentration of ACh is reduced as the agonist concentration is raised. This reduction in the peak response we will continue to call "block," for consistency with previous studies, but no particular mechanism is assumed. Upon removal of agonist, there is a sudden increase in the current. It is clear from the data (Fig. 3) that, after removal of 10 mM ACh, the current rises to a level approaching that after removal of 1 mM ACh, although the response in the presence of 10 mM ACh is less than that in the presence of 1 mM ACh. Upon removal of ACh, the current increases rapidly along a time course that is well fitted by a sum of exponentials (Fig. 3 B). The ability of ACh to block nicotinic channels is significantly reduced at positive potentials, although the blocked channels appear to unblock just as quickly.

The Concentration Dependence of Block

We have examined the concentration dependence of both the degree of block and the unblocking rate. The degree of block is represented by the blocked ratio: the ratio of the number of blocked channels to open channels in the presence of ACh. In practice, we took the ratio (I_b/I_o) of the increase in current after removal of ACh to the current in the presence of ACh (measured just before removal of ACh). This process is described in more detail in Methods. Fig. 4 shows the blocked ratio for both Q-A33 and Q-F18 cells at positive and negative potentials. At a given potential, the blocked ratio obtained from adult-type receptors expressed by Q-A33 cells is similar to that obtained from fetal-type receptors in Q-F18 cells. However, there is clearly a greater degree of block at −60 mV than at +40 mV for both cell types. Note that we will consider here the results obtained by back-extrapolation of exponential fits (Fig. 4, squares and solid lines), as described in Methods.

Simple models of open channel block require that the blocked ratio be proportional to the concentration of the blocking agent (in this case ACh itself). To test this hypothesis, we fitted the data of Fig. 4 with y = ax + b, where x is the logarithm of the ACh concentration and y is the logarithm of the blocked ratio. If the blocked ratio is proportional to the agonist concentration, then we would expect to find a unit gradient for the data plotted in Fig. 4. Fits to the data (Fig. 4, solid lines) deviate from a = 1, the expected result, under all conditions.
The fitted parameter values are given in Table I. The deviation from unit slope is most apparent at low concentrations (Fig. 4, D and E) and positive potentials (Fig. 4, A, B, and C).

We have taken the unblocking rate to be the rate constant of the exponential component describing the rapid increase in current upon removal of ACh. The unblocking rates measured from many patches of both Q-A33 and Q-F18 cells are plotted in Fig. 5. Although the unblocking rate after application of 3 mM ACh is indistinguishable from the rate after 10 mM ACh, there is a slight tendency for the...

FIGURE 4. The proportion of channels blocked by ACh, as a function of ACh concentration. Graphs A, B, and C show the blocked ratio I_b/I_o (the ratio of blocked to unblocked channels in the presence of high ACh concentrations) at a membrane potential of +40 mV, for cells expressing either adult-type (A and B) or fetal-type (C) subunits. Graphs D, E, and F show the same at a membrane potential of -60 mV. Graphs A and D show data from a single cell (the same data are displayed in Fig. 3). The current just before the removal of ACh from an outside-out patch is termed I_o. The current immediately after the removal of ACh is I_o plus that part of the current (I_b) previously obscured or removed by the blocking action of ACh. I_o and I_o+I_b were obtained from data traces similar to those shown in Fig. 3 and were estimated in two ways, as described in Methods: as the mean current over time chosen just before and just after the removal of ACh (I_o/I_o plotted with triangles) and by fitting the section of data after the removal of ACh with a sum of exponentials and calculating I_o+I_b from the amplitudes and the equilibrium current. I_o+I_b in this case is plotted with squares. Up to eight measurements at different ACh concentrations and potentials were obtained from each patch. To test the hypothesis that the blocked ratio (I_b/I_o) is proportional to ACh concentration, the data were represented as a log-log plot and fitted with the function ax + b, where x = log_{10}[ACh]. The data were weighted by the number of measurements per point. The parameters a and b are given in Table I.
rate after application of lower concentrations to be slower. We do not think that this is a real trend, because it is in many cases difficult to align the small changes obtained with low ACh concentrations, and as a consequence, the average tends to be more spread out in time. This will lead to an underestimation of the unblocking rate. In passing, we mention that poorly aligned data would also lead to an error in the measurement of the blocked ratio by back-extrapolation. To avoid this, we used data for the blocked ratio only if the measured unblocking rate exceeded the arbitrary value of 29,000 s⁻¹.

In contrast to the degree of block, the unblocking rate seems unaffected by changes in the transmembrane potential. There is quite a variation in measurements of this rate, but most lie between 20,000 and 55,000 s⁻¹. To support the premise that the rate of recovery of the patch current from block by ACh is not significantly restricted by the finite time that it takes to remove agonist from the patch, unblocking rates measured from the responses of several patches were plotted against the solution exchange times measured from the same patches (Fig. 5 C). Two estimates of the solution exchange time are shown in Fig. 5 C. The first (closed circles) is the rate constant obtained from an exponential fit to the last section of the junction current recorded after the destruction of a patch (see Methods and Fig. 1). In Fig. 8, this would be the section represented by the exponential with a rate of 91,000 s⁻¹ rather than 173,000 s⁻¹. The second (shown by open circles in Fig. 5 C) is a more conservative estimate of the exchange time, obtained from the increase in current that occurs when 100 μM ACh in a low NaCl solution is removed from a patch by the rapid return to normal NaCl bath solution. The increase in current is hypothesized to reflect the increase in permeant ion concentration at the open channel. The solution change is being measured at a point that could hardly be closer to the blocking site and under experimental conditions that are as close as possible to the conditions for recording data. To provide the most direct comparison with the unblocking rate, this response was fitted with the sum of exponentials and the solution exchange rate was estimated from the rate constant of the exponential describing the increase in current upon restoration of normal NaCl bath

<p>| Table 1 Parameters Fitted to the Concentration Dependence of the Blocked Ratio |
|---------------------------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Q33, +40 mV</th>
<th>Q33, -60 mV</th>
<th>Q18, +40 mV</th>
<th>Q18, -60 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0.28 ± 0.13</td>
<td>0.67 ± 0.07</td>
<td>0.41 ± 0.10</td>
</tr>
<tr>
<td>b</td>
<td>-0.09 ± 0.30</td>
<td>1.51 ± 0.18</td>
<td>-0.06 ± 0.24</td>
</tr>
<tr>
<td>$K_0 = 10^{-b/a}$ (mM)</td>
<td>(5,300)</td>
<td>5.6</td>
<td>(1,400)</td>
</tr>
</tbody>
</table>

The data of Fig. 4, obtained by back-extrapolation, were fitted with the equation $y = ax + b$, where $y$ is the logarithm of the blocked ratio, calculated by back-extrapolation of exponential fits to the data on removal of ACh, and $x$ is the logarithm of the concentration of ACh. The fitted values of $a$ are inconsistent with the hypothesis that block is caused simply by an ACh ion directly occluding the channel, which would require that the blocked ratio be proportional to the concentration of ACh and that $a$ therefore would be unity. Values are given as means ± SE. Values of the $K_0$ for +40 mV are in parenthesis because the interpretation of an $x$-intercept as the $K_0$ would in this case be nonsensical.
solution. In Fig. 5 C, it is apparent that the unblocking rate is not correlated with either estimate of the solution exchange rate; therefore, it does not appear plausible that the unblocking rate is limited by the time course of the solution change. In the Discussion, we examine this question in more detail with the aid of simulated solution exchange profiles.

**Figure 5.** The variation of the unblocking rate with prior concentration of ACh. Multieponential fits were made to data such as those shown in Fig. 3. The exponential rate describing the initial fast increase in current upon removing ACh from the patch is plotted as a function of the prior ACh concentration. In A are data from 12 patches taken from Q-A33 cells. In B are data from 19 patches from Q-F18 cells. The transmembrane potential was -60 mV (squares) or +40 mV (triangles). The data from both Q-A33 and Q-F18 cells encompass a wide range, but do not appear to be significantly different. In C, the unblocking rate (ordinate) is compared with two estimates of the solution exchange rate obtained from the same patch (abscissa). The solution exchange rate shown by the open symbols was estimated from responses to 100 μM ACh in low NaCl solution as described in Methods (see Fig. 1 C). To make the comparison more readily, the solution exchange rate was taken to be the exponential rate constant of the component describing the initial increase in the current upon the simultaneous restoration of the normal NaCl concentration and removal of ACh. The solution exchange rate shown by the closed symbols was estimated from the junction current measured from the pipette after destruction of the patch (Fig. 1 B; see Methods). The diagonal line shows equivalent values: if the unblocking process is not limited by the speed of the solution change, then the points will lie to the right of the diagonal line.

**The Voltage Dependence of Block**

Most of the essential features concerning the voltage dependence of block can be gathered qualitatively from the raw data traces shown in Fig. 6. The currents shown...
were all recorded from a single patch in response to short applications of 10 mM 
ACh and with transmembrane potentials varying from -90 to +40 mV.

Essentially, the proportion of channels that are blocked by 10 mM ACh decreases 
as the transmembrane potential goes more positive. In contrast and as we noticed 
before in Fig. 3 B, the unblocking time course does not appear to vary significantly 
at different transmembrane potentials (Fig. 6 B). The largest inward current in the 
presence of ACh is at a holding potential of -60 mV. At more negative potentials, 
the increase in the driving force for the permeant cations is insufficient to compen-
sate for the enhanced degree of block.

![Figure 6](image_url)

**Figure 6.** The voltage dependence of block. A shows current 
recorded from a single outside-
out patch in response to brief 
applications of 10 mM ACh at a 
range of transmembrane poten-
tials. B shows, on an expanded 
time scale, the part of the re-
response immediately after the 
ACh was removed from the 
patch. Overlaid are curves gen-
erated from double or triple ex-
ponential fits to the current in 
the absence of ACh. The trace 
recorded at a transmembrane 
potential of 10 mV is of a spe-
cial interest, since the current 
abruptly decreases rather than 
increases in magnitude upon re-
moval of ACh. A simple explana-
tion for this effect would be that 
the ionic current carried by 10 
mM ACh is a significant part of the whole, with the contribution to the current from other cations 
being much reduced at this potential. Each trace is the average of 10 or 11 individual responses, 
aligned around the time that ACh was removed. Data were collected at the full bandwidth of the 
EPC7 amplifier, digitized at 1 MHz, and digitally filtered for display at 50 kHz (sinc).

The current as a function of voltage for this patch is shown in Fig. 7 A. The closed 
symbols show the current during the ACh application \((I_o)\), and the open symbols 
represent the peak current after removal of ACh \((I_o + I_h)\). The blocked ratio at dif-
ferent potentials is shown in Fig. 7 B for data from five patches taken from two cells. 
The data conform quite well to a straight line on a semilogarithmic plot, suggesting 
that the blocked ratio has an exponential relationship to the transmembrane po-
tential, declining e-fold for 53 mV. The unblocking rate (Fig. 7 C) shows no such 
voltage dependence.

The response to 10 mM ACh at a holding potential of 10 mV (Fig. 6), which is
very close to the reversal potential of +12 mV, appears to be anomalous. At the end of the application of 10 mM ACh, the current, instead of increasing as expected, decreases by ~50%. This abrupt step is 90% complete in ~10 μs (an exponential with a rate constant of 200,000 s⁻¹ would have a 10–90% rise time of 10 μs) and is not well described by an exponential. Attempts to fit the decaying time course with

**Figure 7.** The voltage dependence of the degree of block and the unblocking rate. A shows the peak current before (closed symbols) and just after (open symbols) the removal of 10 mM ACh. These data were taken from the traces shown in Fig. 6 and are from a single patch. The blocked ratio is clearly voltage dependent, as can be appreciated from the difference between the two curves. For each value of the current measured from the data, the transmembrane potential was calculated from the holding potential, the current, and the series resistance (6.7 MΩ). B shows individual measurements of the degree of block of the ACh-induced current in the presence of 10 mM ACh and at a range of transmembrane potentials. The open symbols show data from a single patch, and the closed symbols show data combined from four more patches. The degree of block or blocked ratio was calculated, as in Fig. 4 and in Methods, by fitting a sum of exponentials to the current recorded upon removal of ACh. The data for one patch are shown both with (open circles) and without (open squares) an adjustment for the presence of the 42 pA of ACh-borne ionic current, estimated to be present with this patch. The data shown by closed symbols have also been corrected for an estimated ACh current that varied between patches. The equation \( r = n_0 e^{-E_r/\Delta} \) (shown by the straight line) was fitted to all the data and suggests a blocked ratio of \( n_0 = 0.59 \pm 0.05 \) SE at 0 mV and an e-fold change over \( E_r = 54 \) mV ± 4 SE. C shows the unblocking rate (as estimated from the fitting of sums of exponentials to the current after the removal of ACh) as a function of transmembrane potential. The results were obtained from the same data as used for B. The unblocking rate appears to show little voltage dependence and decreases e-fold over 310 ± 75 mV (SE).
a sum of exponentials yields a rate constant for the step of 80,000–100,000 s\(^{-1}\) (\(N = 2\)), which even though it appears to be an underestimate, is still somewhat higher than has been found for the channel unblocking rate. Neglecting hypotheses that involve complex kinetics at this potential, we are left with the conclusion that at the end of the application of ACh, there is a sudden decrease in the current carried by individual channels. Because the normal external solution and the 10 mM ACh-containing solutions came from the same stock, it is implausible that this effect is an experimental artifact arising from a small difference in the common, permeant cation (\(\text{Na}^+\) and \(\text{K}^+\)) concentration. Our hypothesis is the ACh itself contributes to the current through the nicotinic receptor and that its contribution becomes significant when the current from the other cations in the external solution is reduced. If it is assumed that the ratio of blocked to unblocked receptors at this potential is 0.5 (see Fig. 7 B), then by measuring the current just before and just after the step, we conclude that permeation of the channels by ACh accounts for 42 pA.

This current, although small, does contribute to the net current during the application of a high concentration of ACh. The equilibrium potential of ACh is very positive (since the internal concentration is essentially 0), so the driving force on ACh movement through the channel is assumed to be independent of the holding potential over the range studied. As a gross approximation, it was assumed that the current carried by ACh is independent of the transmembrane potential over the relatively small pertinent range (-10 to +40 mV).

**Discussion**

The data we have presented here show that the simple occupancy–occlusion model of open channel block (Fig. 12, model 1), in which the ion channel is occluded by a blocking molecule, can explain the effects of high concentrations of ACh at transmembrane potentials negative to -60 mV. However, at positive potentials and for concentrations of ACh of 1 mM or less at -60 mV, more block is observed than is predicted.

We have made a direct estimate of the channel unblocking rate (\(\beta'\) in model 1) and find it to be \(\sim 40,000 \text{ s}^{-1}\). However, we did not observe a significant voltage dependence of the unblocking rate, a result that is inconsistent with the one-site, symmetrical barrier model of the voltage dependence of simple open channel block (Woodhull, 1973).

We will estimate the potential sources of error in these measurements and show that these results cannot plausibly be ascribed to artifact. In particular, we will demonstrate, with the aid of simulated solution exchanges, that our fundamental observations are not significantly affected by our experimental solution exchange times. Then we will present alternatives to the simple model of channel occlusion by an ACh\(^+\) ion.

**The Degree of Block**

The blocked ratio, recorded at a transmembrane potential of +40 mV, has a weak dependence on the ACh concentration, with a log slope of rather less than unity (Fig. 4, A, B, and C). This is inconsistent with simple open channel blockade. Even
at −60 mV (Fig. 4, D, E, and F), there appears to be some deviation of the log slope from unity, especially at low agonist concentrations. We need to ask, first, whether this observed deviation could be a consequence of the methods used to measure currents in the raw data, the finite time taken to change solutions, a series resistance error, a cell-to-cell variation, or the current carried by ACh⁺ ions and, second, whether such an observation has been made before.

Calculating the blocked ratio. As we have mentioned in Methods, measuring mean currents before and after removal of ACh leads to a substantial underestimate of the amount of block when little block is evident (i.e., with low ACh concentrations and at positive potentials). However, adopting the method of back-extrapolation of an exponential fit to the current after removal of ACh leads to an overestimate of the amount of block (most apparent again for low ACh concentrations and positive potentials). The true amount of block lies somewhere between these measurements. Nevertheless, it is clear in Fig. 4, A, B, and C (triangles) that the log slope is less than unity at +40 mV, even for the method that underestimates the degree of block. It is clear then that measured deviations of the data from the simple model of open channel block cannot be ascribed to this part of the data analysis.

The solution exchange time. We examine the hypothesis that somehow the combination of the real time dependence of the solution change and a simple model of open channel block might give rise to our data. A smoothed representation of a real solution change profile was obtained by fitting a continuous curve to an averaged junction current record (see Methods). A Runge-Kutta simulation of the membrane current was then performed, using this profile, for a range of concentrations and at two transmembrane potentials (Fig. 8). The blocked ratio was then determined from these calculated traces by measuring peak currents directly, as opposed to fitting the current with exponentials, and is plotted in Fig. 8 as a function of ACh concentration (heavy lines, solid, −60 mV; dashed, +40 mV). The lighter lines give the linear fits to the real data, taken from Fig. 4, B and E, and the symbols represent data from a single patch (Fig. 4, A and D). Again, it is clear that the simple model does not fit the data.

The effects of series resistance. It should be noted that given a series resistance of 6–7 MΩ, the largest currents in Fig. 3 would give rise to a voltage error of 9–12 mV. During analysis, an ohmic series resistance correction was applied before measuring the amount of block, so that the corrected currents before and after removal of ACh are what would have been expected for the holding potential, given the number of channels open. The number of channels open depends on the transmembrane potential, since the degree of block is voltage sensitive. Thus, there will be an error in the measurement of the blocked ratio due to the difference between the transmembrane potential and the nominal potential of −60 or +40 mV. For a 1,000-pA current observed at +40 mV and 1 mM ACh, the real transmembrane potential is 33–34 mV. However, given that the observed voltage dependence of block is on the order of e-fold per 53 mV, such a voltage error would lead only to a 12–14% overestimate of the amount of block for 1 mM at +40 mV. Hence, the deviations from the simple model cannot be explained this way either.

Cell-to-cell variation. In all cases in which data were acquired at a transmembrane potential of +40 mV, data were also acquired at −60 mV. However, only with
some patches were data obtained over the complete concentration range. To eliminate the possibility that variations from one cell to another might give rise to the nonlinear concentration dependence of block at positive potentials, we examined more closely the results obtained over a wide concentration range from single patches. For example, the data of Fig. 3 were all obtained from a single patch. These data are plotted in Fig. 4, A and D. We find that these data do not differ significantly from the averaged data of Fig. 4, B and E, and that deviations from the simple model cannot be explained as cell-to-cell variation.

**Figure 8.** Modeling a real solution change. This figure demonstrates that a simple model of open channel block, combined with a real solution change profile, cannot reproduce the concentration dependence of block that we measure. The bottom trace of A and B shows an averaged junction current upon changing the NaCl concentration at an open patch pipette. The trace has been smoothed by representing it as a series of monoexponentials (see Methods); two of the rates used are identified in the figure. This trace was used to define the solution change profile from which the other traces were synthesized. A shows synthesized responses of a patch to 2-ms applications of 1, 3, and 10 mM ACh at transmembrane potentials of −60 and +40 mV. B shows, on an expanded time scale, the response when ACh was removed from the patch. The unblocking rate constant is 40,000 s⁻¹, and the binding rate constant is $5.7 \times 10^6$ M⁻¹s⁻¹ at −60 mV and $1.0 \times 10^6$ M⁻¹s⁻¹ at +40 mV. C shows the predicted blocked ratio ($I_b/I_o$) as a function of concentration at −60 mV (heavy solid line) and +40 mV (heavy dashed line). The predicted ratio was obtained at half-log unit intervals, so the heavy lines connect individuals points. The symbols show data from a single patch from a Q-A33 cell (circles, −60 mV; triangles, +40 mV). The lighter lines show straight line fits to all data from Q-A33 patches (taken from Fig. 5, A, B, D, and E). All data shown here were obtained from measuring current amplitudes directly either from averaged data traces or from synthesized data traces. Although this is not the most accurate way to measure the blocked ratio (as we discuss in Methods), it is an entirely transparent approach. As a consequence, the predicted blocked ratio, which should have a log slope of unity for the simple model, falls below a straight line of unit gradient for low ACh concentrations.
The current carried by ACh ions. The small current that we suggest is carried by ACh ions at +40 mV is in the direction opposite to the net cationic current and might be thought to contribute to an apparent excess of block. However, this current would, if anything, be expected to be larger for higher ACh concentrations and hence increase the dependence of the blocking ratio on ACh concentration, rather than decrease it. We therefore discard this hypothesis.

Comparisons with other authors. In data obtained by Ogden and Colquhoun (1985), recorded from frog endplates at −100 to −120 mV and 10−12°C, the mean open time within a cluster has a Hill slope of −0.94 with respect to the concentration of ACh. Although this is closer to unity than our values given in Table I for −60 mV (Q-A33, 0.67 ± 0.07, standard error [SE]; Q-F18, 0.77 ± 0.05), it is quite close to the slope obtained from our data using only values at 3 and 10 mM (Q-A33, 0.90 ± 0.2; Q-F18, 0.88 ± 0.1) and broadly consistent with the simple model. Colquhoun and Ogden (1988) give Ko values for block at −95 to −130 mV that vary a little depending on the model assumed, but are centered around 1.2 mM. Given our measured voltage sensitivity of the blocked ratio (53 mV), this would be equivalent to ~5.5 mM at −60 mV, which is comparable to our value for Q-A33 at −60 mV (5.6 mM). Deviations from a simple model of block are most apparent in our data at positive potentials, so it would be inappropriate to attribute the concentration of ACh producing half-maximal block at +40 mV to the Ko for the blocking step in the simple model. If the blocked ratio at +40 mV, for Q-A33 cells, were to become linear with concentration >10 mM, then the concentration of ACh that produces half-maximal block would be ~25 mM, which is comparable to 25−33 mM at +85 to +120 mV (Ogden and Colquhoun, 1988). We also note a difference between our data and those of Colquhoun and Ogden (1988). If it is assumed that the single-channel conductance is approximately ohmic in the range −60 to +40 mV, then a cursory inspection of the amplitudes of the currents displayed in Fig. 3 will reveal that at the peak of a response to 1 mM ACh, a similar number of channels are open at the two potentials. This is in contrast to the very different maximum open probability values obtained by Colquhoun and Ogden (1988), which are 0.9 at 100 μM ACh, −90 mV, and 0.41 at 500 μM ACh, +40 mV.

The literature is not yet overburdened with studies of the concentration dependence of block by ACh at positive potentials. However, we have been able to extract some more information from a study of the voltage dependence of the mean current during a "high concentration cluster" at various ACh concentrations (Sine and Steinbach, 1984). The predicted depression of the mean current in a cluster (shown in Fig. 11 A, column ii, for two potentials) is not quite equivalent to the blocked ratio. When activation steps are saturated, the mean current in a cluster is reduced from the single-channel amplitude by block and by a component contributed by activation gaps (α/β in the case of model 1). The Sine and Steinbach (1984) data are shown with a correction for the contribution α/β = 0.025 in Fig. 11 A, column iii (closed symbols). It is apparent that the correction for activation gaps does not make the single-channel data equivalent to our data and that the data for +40 mV do not lie on the line (dashed) predicted by model 1; rather, there is more block observed at +40 mV in 1 mM ACh than would be predicted.
The Channel Unblocking Rate

By following the time course of the response of a patch upon removal of high ACh concentrations and averaging the exponential rate constants obtained from many patches, we obtained estimates of the channel unblocking rate for the simple open channel block model of 40,000 ± 2,600 s⁻¹ (SE; N = 20) Q-F18 and 39,000 ± 3,500 s⁻¹ (N = 11) for Q-A33 cells. In addition, we made the perhaps unexpected discovery that the unblocking rate is only slightly voltage sensitive (see Fig. 7 C). As before, we first ask whether our results could be artifactual and then whether they are consistent with previous observations.

A finite time for the conformational change. It is tacitly assumed that when a single-ion channel opens, the current passed by the channel rises instantaneously to the full single-channel current (but see Ferguson, MacManus, and Magleby, 1993). If this assumption were incorrect, then this might be a mechanism for limiting the rate of increase in the current on the removal of ACh. The single-channel current for these receptors follows a time course that is indistinguishable from the system step response at the full bandwidth of the patch-clamp amplifier (10-90% rise time of ≈ 4 μs or less; Maconochie, Fletcher, and Steinbach, 1995), so the rate of increase in current does not appear to be limited by the rate at which the current flowing through an open channel can change.

The solution exchange time. In Fig. 8, we show simulated responses of an outside-out patch to applications of ACh, generated assuming a simple open channel block mechanism and a concentration change profile obtained from the junction current time course at an open pipette (see Methods). The unblocking rate obtained from an exponential fit to these responses ranged from 35,000 to 37,000 s⁻¹. These values are not significantly different from the unblocking rate of 40,000 s⁻¹ fixed in the model.

We also performed more extensive tests using synthesized solution exchange profiles with different time courses. Sigmoidal solution change profiles were synthesized (see Methods) with rise times (10–90%) that ranged from 1 to 100 μs. The simple open channel block model was assumed once more and was used to generate the responses to 10 mM ACh as two parameters were varied: the solution exchange time and the channel unblocking rate (the blocking rate was also varied to keep the equilibrium blocked ratio constant). In addition, we simulated the response of a patch to 100 μM ACh in low NaCl solution followed by removal of ACh in normal NaCl solution (again with a range of solution exchange times). These simulated responses were used to obtain an indication of the solution exchange time comparable to the data of Fig. 5 C. We first assume that the channel unblocking rate is faster than we can measure (160,000 s⁻¹). Fig. 9 C shows the result of using such a value for the unblocking rate in a simple open channel block model (solid line). All but two of the real data points (taken from Fig. 5 C) lie to the right of this curve. This is strong evidence for the idea that the unblocking rate is not as fast as 160,000 s⁻¹. The same approach with the unblocking rate set to 80,000 s⁻¹ gives much the same result (Fig. 9 C, dashed line). This time, three data points lie to the left of the curve. Only when the unblocking rate is set to 40,000 (Fig. 9 C, dotted line) or less do the real data points appear to be evenly scattered to either side of
the theoretical curve, thus supporting the idea that the unblocking rate is 40,000 s$^{-1}$. Of course, these tests rely on the assumption that the simple open-channel model of block is appropriate, and we have already argued against this. Nevertheless, the simple model remains a good approximation of the behavior of these channels at potentials negative to $-60$ mV and for high ACh concentrations.

We can therefore discard the hypothesis that the solution exchange time contributes significantly to the rate of the recovery from block.

**Figure 9.** Modeling combinations of solution exchange times and unblocking rates. A shows the result of modeling simple open channel block in conjunction with a range of solution exchange profiles (10–90% rise times ranging from 1 to 100 μs). The traces show the predicted response of a patch to 2-ms applications of 10 mM ACh. The response to an application with a fast rise time is a current that increases abruptly, reaches a plateau, and then increases abruptly again upon removal of agonist. The large overshoot on the initial application of agonist has been attributed to the development of block as the agonist concentration increases relatively slowly, reaching a blocking concentration only some time after reaching a level that activates most channels (Liu and Dilger, 1991). A small fast overshoot will also be apparent if, as is the case modeled here, the unblocking rate is slower than the channel opening rate. We assumed values of 60,000 s$^{-1}$ for the channel opening rate (D. J. Maconochie and J. H. Steinbach, manuscript in preparation) and 40,000 s$^{-1}$ for the unblocking rate. B shows, on an expanded time scale, the response when ACh was removed from the patch. Also synthesized were responses of a simple open-channel block model to coapplications of 100 μM ACh and low NaCl, using the same set of solution exchange profiles (data not shown). The exponential rate constant fitted to the increase in current upon removal of 10 mM ACh is taken to be an estimate of the unblocking rate, and the rate constant fitted to the increase in current upon replacement of the normal NaCl concentration together with removal of 100 μM ACh is taken to be an estimate of the solution change rate. The estimated unblocking rate is plotted against the solution change rate in C, with the unblocking rate $k_B$ set to 160,000 s$^{-1}$ (solid line), 80,000 s$^{-1}$ (dashed line), and 40,000 s$^{-1}$ (dotted line). Also shown are the data from Fig. 5 C (unblocking rates measured from currents recorded from QA33 cell patches) plotted as a function of the estimated solution change rate (closed symbols, taken from the junction current at the open patch pipette; open symbols, taken from a combined application of 100 μM ACh and low NaCl). Only with the unblocking rate set to 40,000 s$^{-1}$ do the predictions of the model resemble the data, indicating that measurement of the unblocking rate is not restricted by slow solution changes.
Comparisons with other authors. Ogden and Colquhoun (1985), measuring the variance of the current during single-channel events at the frog neuromuscular junction, made an estimate of the unblocking rate of 56,000 s\(^{-1}\) (−125 mV, 10−12°C). Sine and Steinbach (1984) reported a preliminary estimate of the unblocking rate as 40,000 s\(^{-1}\) (Single-channel closed durations attributed to "block," BC3H1 cells, −130 mV and 11°C). We regard these findings as comparable to our data. The low voltage dependence of the measured rate of recovery from block is quite striking (Fig. 6 and Fig. 7 C). Our low voltage dependence is in contrast to observations made for charged local anesthetics (Neher and Steinbach, 1978) and agonists such as suberyldicholine (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985). It is, however, worth noting that no substantial voltage dependence is seen even at negative potentials (Fig. 7 C), at which our observations otherwise most closely resemble the "traditional" model of open channel block.

Models for Channel Block

A series of models were tested for their ability to predict (1) the blocked ratio as a function of ACh concentration at the two transmembrane potentials used here, (2) the unblocking rate as a function of transmembrane potential, and (3) the single-channel cluster mean currents obtained by Sine and Steinbach (1984) as functions of both ACh concentration and transmembrane potential. Qualitatively, the important observations requiring explanation are the relatively weak dependence of the blocked ratio on ACh concentration (especially at +40 mV), the weak dependence of the unblocking rate on transmembrane potential, and the strong dependence of the current on both the ACh concentration and the transmembrane potential. We also consider whether the current observed near the reversal potential, which we hypothesize is carried by ACh\(^+\) ions, is predicted by the reaction rates of our blocking models or whether, as Fatt (1950) suggested, ACh\(^+\) ions permeate the channel. Since the Sine and Steinbach (1984) data are the most complete of any available for this receptor, we began by requiring that all models examined here reproduce the voltage and concentration dependence of the Sine and Steinbach data Fig. 11, columns i and ii) and adjusted our rate constants accordingly. It is worth noting that widely disparate models give similar fits to the voltage dependence of single-channel cluster mean amplitudes (Fig. 11, column i). We then examine what the identical model and rate constants would predict for our data. Their data were collected at a different temperature (11°C), which may introduce some quantitative differences, but qualitatively the data should be comparable.

Although we have shown the simple open channel block model to be inadequate on several counts, it is instructive to discuss it first. We will then move to more complex models that better describe the data to try to identify what appear to be essential kinetic features.

Simple open channel block. The simple model of open channel block (Fig. 12, model 1) gives an adequate description for the block we observe at negative potentials and for ACh concentrations >1 mM, but is unable to describe the concentration dependence of the blocked ratio at positive potentials (see the earlier discussion). Neither can simple open channel block describe the voltage dependence of block, as it would have to be assumed that the blocking ion "senses more than the total applied field when it passes through the channel" (Sine and Steinbach, 1984).
To describe this effect in the succeeding discussion, we will take the electronic charge on the ACh\textsuperscript{+} ion to be constant (unity) and talk about a total electrical distance. So, for example, if for a given model, the best fit of the voltage sensitivity of block requires a degree of voltage dependence of the individual reaction rates that is twice that which can be accounted for by a single electronic charge traversing the transmembrane field, we will say that the model predicts a total electrical distance of 2.

The voltage dependence of binding and unbinding rates may be modeled by assuming that a ligand is charged (z electronic charges per ion, or valence z) and has to cross energy barriers to reach and leave a binding site (Fig. 10). If some fraction (δ) of an applied electric field (E) must be crossed to reach the top of a barrier, then the rate for that transition is multiplied by a factor \( \exp(-z e E/kT) \) (Eyring and Eyring, 1963), where e is the electronic charge, k the Boltzmann constant, and T is the absolute temperature. Sine and Steinbach (1984) assumed that the AChR has a single binding site and symmetrical energy barriers (Woodhull, 1973) and that ACh can leave the blocking site by passing through the channel. With these assumptions, their data were consistent with a single electronic charge on the ACh\textsuperscript{+} ion moving across 0.8 of the transmembrane electric field to reach its binding site. However, the total electrical distance required is 2.4. This would correspond to distances in our schematic representation (Fig. 10) of δ\textsubscript{A} = 0.4, δ\textsubscript{B} = 0.4, and δ\textsubscript{C} = 0.8.

![Figure 10. Eyring rate theory and a two-barrier model of block. A is a kinetic model showing the simple open channel model of block. B is the blocking ion, and R the receptor. An asterisk indicates that the receptor is open. B shows the putative free energy of the system as a function of the distance of the blocking ion from the site. C shows the additional free energy resulting from the presence of a charge ze (the electronic charge) on the blocking ion and a transmembrane potential E. For example, to get to the blocking site, the blocking ion must cross a barrier that is δ\textsubscript{A}ze E higher in the presence of a transmembrane potential E. As a consequence, the rate constant k\textsubscript{i} is greater by a factor \( \exp(-\delta\textsubscript{B}ze E/kT) \).](image-url)
As Sine and Steinbach (1984) point out, this unreasonable total electrical distance invites further mechanistic speculation: for example, requiring some effect of the transmembrane field on the barrier height, or modeling the channel as a multion pore. The requirement for the total electrical distance can be reduced if we allow the energy barriers to be asymmetrical. Fig. 11 A (column i) shows the fit of an asymmetrical barrier model, with a less unreasonable total electrical distance of 1.3 (parameters in Table II). However, the predicted voltage dependence of the unblocking rate is higher than that for the symmetrical barrier model, changing e-fold over 53 mV. By the choice of a different set of distances δ, with δB = 0, it is possible to have the unblocking rate be relatively voltage insensitive, but only at the expense of a total electrical distance of ~2.0.

The fit of the open-channel block model to the blocked ratio as a function of ACh concentration is notably inadequate (Fig. 11 A, column iii), particularly at +40 mV (triangles). The description of the mean cluster current ratio (Fig. 11 A, column i) is qualitatively more accurate, following loosely the low concentration dependence at positive potentials (Fig. 11 A, column ii, triangles). This can be understood if we consider the effects of activation gaps on the predicted cluster mean current. Overall, simple open channel block fails to describe the concentration de-

### Table II

Parameters Used in Models 1–6

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All values are for 0 mV transmembrane potential. An asterisk marks parameters that were fixed. Unused parameters are marked with a dash. The electrical distances δA, δB, etc. are the fraction of the transmembrane electric field that a blocking ion must pass through to reach the top of an energy barrier (see Fig. 10). In addition, δD is the equivalent change in electrical distance experienced by an ACh⁺ ion bound to a blocking site when a conformational change (isomerization) alters the local electric field.
dependence of block at positive potentials and requires a total electrical distance greater than unity.

Alternatives to Simple Open-Channel Block

Reversible models that involve the complete occlusion of the ion channel by a blocking ion—the channel conductance is reduced to 0 immediately upon occupancy of the binding site—predict a blocked ratio that is linearly dependent on ACh concentration (a log slope of unity). Two alternatives were explored to obtain
The predictions of alternative models, concentration and voltage dependence. In the leftmost column (i), we show with permission, the data of Sine and Steinbach (1984). The voltage dependence of the mean current in a cluster of single-channel openings recorded from BC3H1 cells is shown for ACh concentrations of 10 mM (triangles), 5 mM (crosses), and 1 mM (open circles). The closed symbols represent the single-channel current in response to 500 nM ACh. Overlaid are the curves predicted from models presented here. Column ii shows data for the fractional reduction in mean cluster current as a function of ACh concentration, extracted from the data shown in column i (data recorded at +40 mV, triangles; −60 mV, squares; mean and SD of three to six measurements). Column iii shows the concentration dependence of the blocked ratio (the ratio of the number of blocked channels to the number of open channels) at +40 mV (squares) and −60 mV (triangles) for Q-F18 cells (data from Fig. 4). For column iii of A, the data from column ii are included; these were made equivalent to the blocked ratio by subtracting α/β = 0.025 from each value. The values assumed for α and β are 1,500 and 60,000 s⁻¹, respectively (manuscript in preparation). The lines overlaid in columns ii and iii show the concentration dependence predicted by models presented here for potentials of +40 mV (solid line) and −60 mV (dashed line). Column iv shows the predicted ensemble relaxation currents at +40 and −60 mV for ACh concentrations of 300 μM, 1 mM, 3 mM, and 10 mM. These were generated by the eigenvalue method described in Colquhoun and Hawkes (1977), and in the case of models 3, 6, and 6A, the results were confirmed by a Monte-Carlo or Runge-Kutta simulation. A summary of the parameters used to generate the simulations is found in Table II.

A slope of less than unity: models with distinct binding and blocking steps, and models in which the channel is only partially occluded by the blocking ion. Having separate binding and blocking steps makes it easier to construct a model in which the unblocking rate is relatively insensitive to changes in the electric field and with appropriate choices of voltage-dependent reaction rates can result in a reasonable
value for the total electrical distance. We also examine how cyclic models can lead to a reduced total electric distance and specifically whether two multisite pore models can predict our data. Partly for the sake of simplicity and partly to maintain consistency between various models, we have assumed the voltage dependence to be due primarily to the interaction of the ACh⁺ ion and the transmembrane field. We describe, at the appropriate places, the effects of assigning some voltage dependence to isomerization steps.

**A single isomerization step, model 2.** Model 2 (Fig. 12) has been drawn with states $A_2R$ and $A_2RB$ placed on the same horizontal level, to suggest a similarity be-

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**Figure 12.** Kinetic schemes used for modeling block. The symbols have their usual meaning: $R$ is the receptor/channel, $A$ is an agonist, and $B$ a blocker. The parameters $k_+$ and $k_-$ are the forward and reverse binding rates for the blocking ion, respectively; $k_t$ is the rate at which blocking ions unbind by passing through the channel; $\alpha$, $\alpha'$, $\beta$, $\beta'$, etc. are the channel closing and opening rates, respectively; and $\beta$ is the concentration of blocker. Note that for all models, the blocker can leave the channel by passing through ($k_t$) or by unbinding to the outside ($k_-$). Only with models 6 and 6A are these rates kinetically distinct. Elsewhere they are combined into $k_-$ = $k_t$ + $k_-$. The asterisk indicates that the receptor is in a conducting form, and the dagger indicates a partially conducting form. The values of the various rate constants and their voltage sensitivities obtained from comparing predictions of the model with the data are given in Table II.
between these two states. The model could equally well have been drawn with $A_2RB$ below $A_2R^*B$.

The equilibrium ratio of blocked to open receptors is $(k_{+1}x_0\alpha')/\beta'(k_{-1} + k_{+1}x_0)$, where the parameters have the usual meanings (defined in Fig. 12). For low concentrations ($k_{+1}x_0 \ll k_{-1}$), this expression is close to linear in the concentration of blocker $x_0$. At high concentrations, it approaches a constant value of $\alpha'/\beta'$. This is clearly in contrast to our data. However, the voltage sensitivity of this model is instructive. If, for simplicity, the isomerization step is assumed to be voltage insensitive, then a good fit (data not shown) may be obtained for the voltage dependence with a total electrical distance of 1.3, as with the simple model previously described. Assuming a total electrical distance of unity results in insufficient curvature in the range 0 to $-150$ mV.

It is necessary to examine more carefully the assumption that the isomerization reaction is insensitive to the transmembrane electric field. It would be quite a coincidence if the transmembrane field, in the vicinity of the channel, were to remain unchanged by the isomerization of the channel to the blocked state. Nevertheless, that is what we have, up until now, assumed to be the case. Suppose then that the transmembrane electric field changes its distribution across the receptor when the channel makes the transition from state $R^*B$ to state $RB$. In that case, even though the blocking ion need not have physically moved, its electrical distance may well have changed. Depending on whether the ion pore blocks/closes around the blocking ion, or closes to the right or left of the blocking ion (see Fig. 10 D), the blocking ion may experience the equivalent of a movement $\delta_0$ through the electric field of anywhere between $-\delta_0 - \delta_0$ and $1 - \delta_0 - \delta_0$. This will produce some voltage sensitivity of the ratio $\beta'/\alpha'$. We tried other fits with different values of $\delta_0$ and a total distance of unity. A distinct improvement in the fit is found with $\delta_0 = 0.2$ (Fig. 11 B, column $i$).

Clearly, making the ratio $\beta'/\alpha'$ voltage dependent has implications for our data. With $\delta_0 = 0.2$, the ratio $\beta'/\alpha'$ can be expected to change by $e$-fold over 120 mV. If $\beta'$ and $\alpha'$ are assumed to be equally voltage sensitive, then $\alpha'$ is expected to increase by $e$-fold over 240 mV, and $\beta'$ is expected to decrease by a similar amount. How $\beta'$ is related to the unblocking relaxation rate measured from ensemble currents upon removal of high concentrations of agonist depends on what values we assume for $\alpha'$, $\beta'$, and $k_{-1}$. The two are similar only if $k_{-1}$ is 10-fold or more greater than $\alpha'$ and $\beta'$. If binding is assumed to be diffusion limited $(10^8 \text{M}^{-1}\text{s}^{-1})$ and $\beta'/\alpha'$ to be a modest 1/10, then $k_{-1}$ varies from $2 \times 10^7 \text{s}^{-1}$ at $-250$ mV to $6 \times 10^8$ at 100 mV. These values for $k_{-1}$ are more than 10-fold higher than values assumed for $\alpha'$ and $\beta'$; thus we can expect the unblocking rate to follow $\beta'$ closely. In Fig. 6 C, we see that the unblocking rate is relatively insensitive to changes in transmembrane potential and, if anything, decreases $e$-fold over 310 mV. So it would appear that a mildly voltage-sensitive isomerization step can predict our data.

To obtain the fit of Fig. 11 B (column $i$), we had to require that when a channel loses it blocking ion, there is a probability of $1.5 \times 10^{-3}$ that the blocking ion will pass through the channel. The unbinding rate is $\sim 1.6 \times 10^7 \text{s}^{-1}$ near 0 mV. However, at equilibrium, relatively few receptors will be in the state $A_2R^*B$, so even though there might be 1,000 channels in a patch, the ionic current predicted is
~0.1 pA. This is not very close to our experimental estimate of the current due to the passage of ACh ions through the channel and is not large enough for "unbinding through the channel" to be a plausible mechanism for the observed current.

In summary, the single isomerization step, model 2, does not give a better fit than model 1 to our measured concentration dependence. It provides a mechanism for accounting for the voltage dependence of block without assuming an electrical distance greater than unity. Moreover, the small voltage dependence required of the unblocking rate appears to be in accordance with our data.

A simple cyclic model, model 3. This model is almost identical to model 2. The only difference is the addition of a step linking \( A_2R \) to \( A_3RB \). Such a minor addition adds a great deal of complexity to the model. Moreover, the model is reversible only if \( k_9 \) and \( k_{a3} \) (the parts of the unbinding rates \( k_{-1} \) and \( k_{-2} \) assumed to be through the channel) are both 0. If \( k_9 \) and \( k_{a3} \) are not 0, the equilibrium values of the degree of block are more complex to calculate, since it cannot be assumed that there will be no net flux around the loop. For each combination of blocker concentration and transmembrane potential, it can be assumed that the flux entering any state is the same as the flux leaving it, so the occupancies of each state can be calculated from a set of three linear equations in the three independent state occupancies.

The rate constants at 0 mV were set according to the following criteria. The two forward binding rates were assumed to be diffusion limited \((10^8 M^{-1} s^{-1})\) as usual, both isomerization steps to the open state were set to 40,000 s\(^{-1}\), the unbinding rate \( k_{a3} \) was adjusted so that the model would be reversible for \( k_9 \) and \( k_{a3} = 0 \), and the remaining rates were freely varied.

It was assumed for simplicity that, in the absence of a blocking ion, the isomerization reaction \((A_2R* \rightarrow A_2R)\) would have no voltage sensitivity. The two binding reactions were assumed to be voltage sensitive, but it was not assumed that the electric field distribution would be the same for each. The voltage dependence of the isomerization to the closed state with blocking ion bound was then adjusted so that the model would be reversible with \( k_9 \) and \( k_{a3} \) set to 0. Finally, the voltage sensitivities of \( k_9 \) and \( k_{a3} \) were independently varied. In all, 10 free parameters were adjusted to obtain the predicted voltage dependence of Fig. 11 C (column i).

At +40 mV, there is very little occupancy of the \( A_3RB \) and \( A_3R^*B \) states. This degree of voltage sensitivity of the binding reactions was necessary to obtain the curvature below 0 mV. As a consequence, the mean cluster current at +40 mV (Fig. 11 C, column ii) is determined mainly by the ratio \( \alpha/\beta \) and does not vary much with agonist concentration (even less so than the data shown) (triangles). In contrast, the predicted blocked ratio (Fig. 11 C, column iii) does not have a contribution from the state \( A_2R \) at limiting low ACh concentrations and falls well below the data for +40 mV. Model 3 does not appear then to be any better at predicting our data.

We also tried a variant of model 3 (model 3A), which is also cyclic but has an additional liganded closed state \( A_2R \) (see Fig. 11). The reaction rates and voltage sensitivity were left unchanged. The predictions of such a model are shown in Fig. 11 D. Not surprisingly, the additional closed state further reduces the predicted mean cluster current at +40 mV (seen as a raising of the dashed line in Fig. 11 D, column ii); however, the predicted blocked ratio at +40 mV (column iii) has now dropped off the bottom of the graph.
We have not mentioned the voltage sensitivity of the isomerization reactions. Despite designing the model with an unblocking rate of 40,000 s$^{-1}$ in mind ($\beta'$ and $\beta''$; see Table I), we discover that for model 3, the rise in the ensemble current upon removal of ACh is governed mainly by the rate of unbinding of the activating ACh ion that is set to 10$^5$ s$^{-1}$. This far from obvious result occurs because there is a direct and fast ($k_1 \sim 3 \times 10^4$ s$^{-1}$) route for closed/blocked channels $A_2R_B$ to reach $A_2R$ and then inactivate. The case for model 3A is different: the rate of recovery from block is now determined mainly by $\beta''$, which over the range $-100$ to 0 mV increases $e$-fold per 90 mV, which of course is in the direction opposite to our data.

Some mechanistic insight can still be obtained from model 3. To get the degree of curvature between 0 and $-60$ mV, $\delta_{1A}$ and $\delta_{1B}$ had to be adjusted to 0, and $\delta_{IC}$ to unity. This suggests that when the channel is closed, the ionically inaccessible portion of the channel does not include this binding site. To get relief from block at very negative potentials, it is necessary either to have a significant passage of blocking ions through the closed channel ($k_{li} = 5,000$ s$^{-1}$), or to have the total electrical distance for the binding reaction to the open channel ($\delta_{2A} + \delta_{2B} + \delta_{2C}$) be greater than unity.

We can estimate the current expected to be carried by ACh ions for a patch with 1,000 channels to be near 0 mV. With the parameters fit in Fig. 11 C, the current would be $\sim 0.3$ pA.

In summary, the cyclic models reproduce the voltage dependence observed for single-channel cluster mean currents (Fig. 11, C and D, column i). Although the concentration dependence of the predicted block ratio at $+40$ mV (Fig. 11, C and D, column ii) is somewhat at odds with our data, the qualitative similarity between the predictions for the mean cluster current and the data in Fig. 11, C and D (column ii), does suggest that a better model would include more than a single blocked state.

The models discussed so far have described, with varying degrees of success, the voltage dependence of the degree of block and the unblocking rate. They have two properties in common: they do not describe the concentration dependence of the blocked ratio at positive potentials, and they assume only a single blocking binding site. We will now move to models that have more than one binding site.

A two-site model of block, model 4. Given the difficulty of dealing with the described cyclic model (the large number of parameters to adjust and the consequences of irreversibility), we chose to work with a branched model in developing a model with two binding sites.

Model 4 incorporates both a low and a higher affinity binding site for block. Both lead to isomerization steps, but it is assumed that in the concentration range modeled, only the higher affinity site will become saturated. High affinity binding is represented by the step $A_2R^* \to A_2R^*B$. For negative transmembrane potentials and high blocker concentrations, most of the block will be due to sojourns in state $A_2R_{B^*}$.

It was assumed for simplicity that only the binding steps are voltage sensitive. As usual, the forward binding rates were set to 10$^3$ M$^{-1}$ s$^{-1}$, and the blocking rates $\beta'$ and $\beta''$ were set to 40,000 s$^{-1}$. The results of manipulating six electrical distances,
two unbinding rates, and the two ratios ($\beta'/\alpha'$ and $\beta''/\alpha''$) are shown in Fig. 11 D. Model 4, like model 2, has been drawn with states $A_2R$, $A_2RB$, and $A_2RB_2$ on the same horizontal level, again to suggest a similarity between these states.

With this model, it is possible to obtain a very close fit to the voltage dependence of block, to the reduction in the mean cluster current, and a qualitatively reasonable approximation of the blocked ratio. The total electrical distance for the high affinity binding step was a reasonable 0.5, and that for the low affinity binding was a less fortunate 1.3. We would not care to draw any mechanistic inferences from the precise values of the electrical distances obtained, since widely differing combinations of the 10 parameters can give quite similar results. Nor do we mind that the total electrical distance exceeds unity, since we have previously shown that allowing the isomerization steps to be mildly voltage sensitive or making the model cyclic by allowing additional transitions (for example, between the two closed or blocked states $A_2RB$ and $A_2RB_2$) can compensate for this defect. Owing to the very low equilibrium occupancy of state $A_2R*B_2$, the current carried by $\text{ACh}^+$ ions as a result of unbinding through the channel is minuscule.

In summary, this model reproduces almost every aspect of our data, but at the expense of some simplicity in the model. A more complex model based on this model might fit the data better, but would be intractable.

A two-site, partial occlusion model of block, model 5. This model is similar to model 4 in having two binding sites for blocking ions. The difference is in the mechanism by which binding to the first blocking site causes only a partial reduction in the mean current. Instead of having an isomerization reaction to a closed state (but favoring the open state), it is assumed that the blocking ion only partially occludes the conducting pore. This is represented in Fig. 12 by $R_t^t$, denoting a subconductance state of $R$. We tried a range of different subconductance levels, but the best agreement was obtained with only a 10% reduction of the single-channel amplitude for state $R_t^t$.

For this model, relief from block occurs when the blocking ion unbinds, so both unbinding rates were set to 40,000 s$^{-1}$ and were made voltage insensitive by setting $\delta_{1B}$ and $\delta_{2B}$ to 0. The affinities of the two binding reactions were adjusted by varying the two forward binding rates ($k_{+1}$ and $k_{+2}$).

This model reproduces quite well both the voltage and concentration dependences of the mean cluster current. There is also good qualitative agreement with our data, as would be expected given the similarities between this and model 4.

Given that we assumed the same mechanism for block as model 1 (simple open channel block), we would expect the voltage dependence of the blocking and unblocking rates for the complete occlusion step ($k_{-2}$ and $k_{-2}$; Table II) to be comparable. We found that if we made the unblocking rate $k_{-2}$ voltage insensitive, then the total electrical distance is 2.02 (as for model 1). It was necessary to assume a small voltage dependence of the blocking reaction that produces the subconductance state ($\delta_{1A}$). This would be compatible with a binding site near the outside of the channel pore. The very small value of the unblocking rate through the channel, $k_{2A}$, predicts an immeasurably small current component due to the passage of $\text{ACh}^+$ ions.

The major advantage of this model is that it uses a minimum number of states...
and parameters in making accurate predictions of the concentration and voltage
dependence of block. The main limitation is that the total electrical distance is
greater than unity.

A multisite, single-ion pore model, model 6. Model 5, the partial occlusion model,
has some aspects that are consistent with a view of the channel as being able to con-
tain more than one ion at a time: a multi-ion pore. It would not, however, be a sin-
gle-file pore, since occupancy by a blocking ion of one of the ion-binding sites only
partially reduces the throughput of the usual permeant ion. Although Levitt
(1986) concluded that most evidence was in favor of the nicotinic receptor being a
single ion pore, the likely quaternary structure is not entirely compatible with this
view.

We consider here only a very simple model with two blocking sites in the channel
pore. It is assumed that when either site is occupied, no other ionic species may
pass through the channel. A schematic picture of this model is given in Fig. 13. Spe-
cific interactions between blocking and permeant ions are neglected (we assume
that their effects on the voltage dependence of block may be approximated by ma-

Figure 13. A schematic description
of models 6 and 6A. The picture shows
the ACh receptor in the various con-
formations assumed to be related to
the discrete kinetic states of model 6A.
Two ACh ions are shown bound to
sites external to the pore before the
channel can open, and binding to two
additional sites in the ion channel
pore itself are responsible for the
blocked states $A_2BR$, $A_2RB$, $A_2BRB$.
Model 6 is identical except that the
state $A_2BRB$ does not exist.

nipulation of the electrical distances). It is not assumed that the two sites are also
sites for the usual permeant cation, although this would be the simplest interpreta-
tion of the reduction in current when a site is occupied. We are examining the abil-
ity of such a model to reproduce the concentration dependence of block. We con-
sider first the case in which, although there are two sites in the channel for the
blocking ion, it is unlikely that both are ever occupied simultaneously (model 6)
and then the case in which binding to either site is unaffected by occupancy of the
other site (model 6A).

Model 6 may be described as follows: an ACh ion may enter the channel and
bind to the first binding site, thereby blocking the channel ($A_2R^*$ to $A_2BR$). The
blocking ion can then either leave the channel, or move to the second site ($A_2RB$).
From the second binding site, the blocking ion can also pass through the channel.

Since for this model, only the electrical distance between the two energy wells
forming the two binding sites for the blocker is important, the wells were assumed
to be equidistant (δ0) from the barrier peak separating them. The rates for crossing this barrier were set, somewhat arbitrarily, to large and equal values.

This pleasingly simple model predicts very well the voltage and concentration dependence of the Sine and Steinbach (1984) data (Fig. 11 G, columns i and ii), but not so well our data at positive potentials (Fig. 11 G, column iii). The total electrical distance is 1.3, and the predictions for the electrical distance of the energy wells or binding sites from the external end of the channel are 0.05 and 0.95.

Also of note are the predicted ensemble current traces of Fig. 11 G (column iv), which do not at all resemble our data. First, block appears to develop slowly (the current sags after the start of the agonist application); second, the recovery from block is also remarkably slow given the chosen rate constants. The shape of these data (and those for model 6A; fig. 11 H, column iv) were sufficiently unexpected that we suspected some defect in the eigenvalue routine used to generate the ensemble current predictions. So we repeated the synthesis of the data in Fig. 11 G (column iv) and H (column iv) using a Runge-Kutta numerical routine (as described in Methods), with identical results. The origin of this counter-intuitive result is the unbinding of ACh through the channel, which enables the reaction model to cycle irreversibly.

The relative simplicity of this model is its only advantage. The discrepancy with our data at positive potentials is too glaring. Moreover, given the very low rate of unbinding of ACh ions through the channel (55 s⁻¹; Table II) and our measured ACh-associated current of 42 pA, we have to conclude that the ACh-binding sites in this model are not the "normal" cation-binding sites associated with a conducting pore model.

A two-site, multi-occupancy model, model 6A. This model is similar to model 6; the only difference is that a second ACh⁺ ion can bind when the first ion has moved to the second binding site. For simplicity, the rate constants for binding and unbinding to the first site are the same, whether or not an ACh⁺ ion is in the channel. In addition, the rate for going through the channel is also assumed to be independent of the number of ACh⁺ ions in the channel. The isomerization rates are fixed as before.

This model also predicts well the voltage and concentration dependence of the Sine and Steinbach (1984) data (Fig. 11 H, columns i and ii), but does not predict so well our data at positive potentials (Fig. 11 H, column iii). The log slope for positive potentials is too steep. The total electrical distance is a little greater than that for model 6 (1.40). The energy wells are predicted to be at electrical distances 0.2 and 0.8 from the external end of the channel.

As with model 6, the ensemble current predictions contain unexpectedly slow relaxations for both the development of and the relief from block. However, it is not possible to exclude this model, since only a limited range of reaction rates were tested.

**SUMMARY AND CONCLUSIONS**

We have used fast agonist applications to follow block by ACh of the nicotinic receptor. We find that the unblocking rate is ~40,000 s⁻¹ and is relatively insensitive
to the transmembrane potential. At negative potentials and high ACh concentrations, block otherwise conforms broadly to earlier concepts of open channel block.

We find that to explain the deviations we see at low ACh concentrations and positive potentials, we need to include in our model two ACh-binding sites associated with block, with different affinities. Of the models that we have examined, models 4 and 5 predict our data the most closely, the model 6A might do so if we were better able to pick appropriate rate constants. However, there are caveats with each model. Model 5, for example, is pleasingly simple in concept, but requires an additional mechanism to explain the large electrical distances involved. Moreover, with model 5, the unblocking rate can have only a positive relationship to the transmembrane potential (we observe a decrease of e-fold over 310 mV). Model 4 is capable of predicting every aspect of our data. With minor adjustments, it can also predict the voltage dependence of the unblocking rate, while keeping the total electrical distance to a reasonable value. This is, however, at the cost of two additional kinetic states. Our conclusions, therefore, must be that block by ACh of the nicotinic channel is more complex than had previously been envisioned and that none of the restricted models that we have examined is the best predictor of our data. The truth may be that aspects of several of the models may play a role: two binding sites, a partially conducting state, additional isomerization steps, and multiple blocking ion occupancy of the conducting pore.

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REFERENCES

Fatt, P., and B. Katz. 1951. An analysis of the end-plate potential recorded with an intra-cellular elec-


Sine, S. M., and J. H. Steinbach. 1984. Agonists block currents through acetylcholine receptor chan-
Maconochie and Steinbach  Nicotinic Receptor Block by Acetylcholine 147