Curare binding and the curare-induced subconductance state of the acetylcholine receptor channel

George Joseph Strecker* and Meyer B. Jackson‡
Departments of *Physiology and ‡Biology, University of California, Los Angeles, California 90024-1601

ABSTRACT The curare-induced subconductance state of the nicotinic acetylcholine receptor (AChR) of mouse skeletal muscle was examined using the patch-clamp technique. Two mechanisms for the generation of subconductance states were considered. One of these mechanisms entails allosteric induction of a distinct channel conformation through the binding of curare to the agonist binding site. The other mechanism entails the binding of curare to a different site on the protein. Occupation of this site would then limit the flow of ions through the channel. The voltage dependence and concentration dependence of subconductance state kinetics are consistent with curare binding to a site within the channel. The first order rate constant for binding is 1.2×10^6 $M^{-1}s^{-1}$ at 0 mV, and increases e-fold per 118 mV of membrane hyperpolarization. The rate of curare dissociation from this site is 1.9×10^2 s^{-1} at 0 mV, and decreases e-fold per 95 mV hyperpolarization. The equilibrium constant is 1.4×10^{-4} M at 0 mV, and decreases e-fold per 55 mV hyperpolarization. This voltage dependence suggests that the fraction of the transmembrane potential traversed by curare in binding to this site is 0.46 or 0.23, depending on whether one assumes that one or both charges of curare sense the electric field. Successive reduction and alkylation of the AChR agonist binding sites with dithiothreitol (DTT) and *N*-ethyl maleimide (NEM), a treatment which results in the loss of responsiveness of the AChR to agonists, produced no change in curare-induced subconductance events, despite the fact that after this treatment most of the channel openings occurred spontaneously. Mixtures of high concentrations of carbamylcholine (CCh) with a low concentration of curare, which produce channel openings gated predominantly by CCh, resulted in subconductance state kinetics similar to those seen in curare alone at the same concentration. Thus displacement by CCh of curare from the agonist binding sites does not prevent curare from inducing subconductances. The results presented here support the hypothesis that curare induces subconductance states by binding to a site on the receptor other than the agonist binding sites, possibly within the channel pore. It is the occupation of this site by curare that limits the flow of ions through an otherwise fully opened channel.

INTRODUCTION

The nicotinic acetylcholine receptor channel (AChR) is an allosteric protein which undergoes a conformational change resulting in the opening of a transmembrane pore to the flow of cations. The duration and uniform magnitude of these unitary events is readily seen with the patch-clamp technique (Hamill et al., 1981). However, single-channel recording has shown that this channel can also assume one or more levels of lower conductance, which may appear in association with a full conductance state. Such subconductance events have been seen in patch clamp recordings from preparations such as embryonic rat muscle with acetylcholine (ACh) as agonist (Hamill and Sakmann, 1981), cultured embryonic chick muscle with carbamylcholine (CCh) or ACh as agonists, (Auerbach and Sachs, 1983, 1984), and purified and reconstituted AChR from *Torpedo* with ACh as agonist (Tank et al., 1983). GABA (gamma-aminobutyric acid) and glycine receptor channels (Hamill et al., 1983) and glutamate receptor channels (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987) also display multiple-conductance state behavior. In addition, multiple-conductance state behavior is seen in channels formed by the peptide antibiotics alamethicin (Eisenberg et al., 1973) and gramicidin (Busath and Szabo, 1981) in lipid bilayers.

Subconductance events are also seen when the nicotinic AChR is gated by *d*-tubocurarine (curare). The role of curare as a competitive antagonist of the nicotinic AChR is well established (Jenkinson, 1960), but there is evidence that it acts as a noncompetitive blocker of the open channel as well (Marty et al., 1976; Manalis, 1977; Katz and Miledi, 1978; Colquhoun et al., 1979; Trautmann, 1982; Takeda and Trautmann, 1984). In addition, curare has the properties of a weak agonist of the AChR in some preparations (Ziskind and Dennis, 1978; Jackson et al., 1982; Trautmann, 1982; Morris et al., 1983). These findings suggest that curare interacts with two functionally distinct regions of the AChR, the channel pore and the agonist binding sites. In addition to giving rise to the full conductance which is characteristic of the nicotinic

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AChR, curare induces a subconductance state in these channels in cultured rat myotubes (Trautmann, 1982), the mouse skeletal muscle cell line G-8 (Morris and Montpetit, 1985), and cultured mouse myotubes (Strecker and Jackson, 1988).

Hypotheses for the mechanism of subconductance stage generation in the AChR can be classified broadly into two distinct groups. One class of hypotheses proposes that the AChR complex can undergo a change in conformation which results in an open channel with reduced conductance (Hamill and Sakmann, 1981; Morris and Montpetit, 1985). Alternate hypotheses propose that the subconductance state represents a fully open channel whose conductance is reduced by an exogenous agent, which binds to the channel protein and interferes in some way with ion permeation, rendering the channel partially blocked. The exogenous agent that induces this partial block could be an agonist molecule that binds to a site within the channel pore. Takeda and Trautmann (1984), examined the dependence of subconductance behavior on curare concentration and membrane potential, and concluded that the partial-block hypothesis was slightly favored.

The possibility that curare may both activate the AChR and occlude its channel complicates the study of interactions of this ligand with the receptor. Curare might produce a subconductance state by binding to a site other than the two agonist binding sites of the AChR, and reduce ion flow through the channel pore without inducing any conformational change. If this is true, experimentally altering the agonist binding sites should not affect subconductance behavior. Alternatively, if curare elicits a subconductance state allosterically, it might do so by acting through the agonist binding sites. Here we investigate these possibilities by modifying the interaction of curare with the AChR agonist binding sites in two different ways.

The AChR contains two agonist binding sites, one on each α-subunit (for review see Karlin, 1980; Popot and Changeux, 1984). Binding of agonist to these sites induces opening of the associated ion channel. Karlin and Bartels (1966) showed that the AChR-mediated depolarizing response of the *Electrophorus* electroplax to CCh could be reduced to 12% of its initial amplitude, by reducing labile disulfide bonds with dithiothreitol (DTT) and alkylating these reduced sites with *N*-ethyl maleimide (NEM). Numerous subsequent studies have confirmed the ability of this reduction and alkylation treatment to reduce the responsiveness of the AChR to various agonists (for review see Karlin, 1980). Such modification results in an apparent decrease in the affinity of agonists for the AChR (Karlin, 1969; Walker et al., 1981), and is thought to occur very near the agonist binding sites on the α-subunit (Kao et al., 1984). However, DTT-NEM treatment interferes neither with the ability of the channel to open spontaneously, nor with the open channel conductance (Jackson, 1984). Thus, this treatment provides us with a means of altering the agonist binding sites without perturbing the ion channel.

Another means of modifying the interaction of curare with the agonist binding sites of the AChR is to displace curare competitively from these sites with high concentrations of another agonist which produces subconductance events at a negligible rate. We demonstrate here that displacing curare from the agonist binding sites does not influence the kinetics of subconductance state occurrence. Taken together with the concentration and voltage dependence of this phenomenon, these findings support the hypothesis that the curare-induced subconductance state is produced by a mechanism of partial channel block, which does not involve the agonist binding sites.

MATERIALS AND METHODS

Preparation and treatment of cultured cells

The procedures for preparing primary cultures of mouse thigh muscle were identical to those used in previous studies in this laboratory (Jackson, 1986; 1988). Recordings were made from muscle fibers in 6–10-d-old cultures.

For electrophysiological recording, the growth medium was removed and the cells were bathed in a physiological saline solution consisting of 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 15 or 20 mM glucose, 1 μM tetrodotoxin, and 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.3). Reduction and alkylation of the AChR was achieved by first bathing the cells in this saline containing 1 mM DTT for 20 min, and then exchanging this solution for saline containing 1 mM NEM. After 10 min, the NEM was washed out and replaced with fresh saline. Electrophysiological recording was initiated immediately after treatment. Cells treated in this way will be referred to as DTT-NEM treated cells.

Data recording and analysis

Single-channel currents were recorded in the cell-attached configuration (Hamill et al., 1981) using an EPC-7 patch clamp (Medical Systems, Great Neck, NY). Patch electrodes were pulled from 1.5 mm o.d., 0.75 mm i.d. borosilicate glass capillary tubing (Garner Glass Co., Claremont, CA), and coated with Sylgard (Dow Corning Corp., Midland, MI) to reduce capacitive noise. The electrodes were filled with the physiological saline described above and were clamped at various potentials ranging from 0 mV to +125 mV (hyperpolarizing the cell-attached membrane patch). Assuming an AChR reversal potential of 0 mV and a linear current-voltage relation, the full state conductance was 39 pS. Because we did not monitor the cell resting potential directly, we used the amplitude of the channel currents and the 39 pS conductance value to estimate the driving force (equal to the resting potential minus the pipette voltage), and thus the total membrane potential V_m. This gives a better estimate of the driving force than assuming some value for resting potential. All the membrane potentials given here are estimated in this way.

Membrane current was filtered at 10 kHz (EPC-7 filter), digitized with a modified digital audio adapter (Sony PCM-501 or 601, Unitrade, Philadelphia, PA) and stored on β-format videocassette tape, as described by Bezanilla (1985). Appropriate sections of the recorded data were prepared for analysis by re-filtering with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) at a final bandwidth of 3 or 5 kHz. Automated analysis of the subconductance state was most accurate with data filtered at 3 kHz. The full conductance state, due to the larger current, could be analysed in data filtered at 5 kHz, for comparison with previous studies utilizing the same bandwidth (Jackson, 1988). Data were subsequently sampled with an A/D converter (Data Translation DT 2782-DI/A), and written onto the hard disk of an LSI-11/23 computer at 24 or 40 kHz sampling rate for 3 or 5 kHz bandwidth data, respectively.

Using automated channel event detection software described elsewhere (Jackson, 1986; 1988), channel current records were converted into idealized lists of amplitudes and durations. Events that were too brief to measure accurately (i.e., shorter than 200 μs for 3 kHz data or 113 μs for 5 kHz data) were rejected. Additional programs were written to display the amplitude histograms, count subconductance transitions, and estimate the time spent at various conductance levels. Automated analysis was tested by comparison with manual analysis of several data sets. Average fully open channel and baseline amplitudes were picked by eye as peaks in the amplitude distributions. For counting subconductance events, the ranges of amplitudes encompassing the full and subconductance states were determined automatically by the transition counting program, in proportion to the full channel size. These ranges were made sufficiently narrow to minimize errors introduced by the overlapping of subconductance and fully open events. When counting the number of subconductance events, only those with transitions to or from the full conductance state were counted, to minimize the influence of spurious low amplitude events.

Channel open times of the isolated full conductance state (subconductance states excluded) were fitted to a probability density function of the form

$$f(t) = \sum_{i=1}^{m} x_i \alpha_i e^{-\alpha_i t} \quad (1)$$

using maximum likelihood methods described elsewhere (Horn and Lange, 1983; Jackson, 1986; 1988). α_i represents the decay constant of each exponential and x_i represents the relative area of each of the m exponentials. These open time distributions were corrected for missed events due to short and long time cutoffs of 0.113 and 10.0 ms, respectively, by the expression (Colquhoun and Sigworth, 1983)

$$N = N_0 \int_{ts}^{tl} x_i \alpha_i e^{-\alpha_i t}, \quad (2)$$

where the limits are the short and long open time cutoffs, N_0 is the total number of events expected at infinite bandwidth, and N is the actual number of events counted within the specified range. With records showing high levels of channel activity, curve fitting methods appropriate for a many channel system were used (Jackson, 1985, 1988).

Comparisons of means between two or between several groups of data were evaluated for significance using the T-test or analysis of variance (ANOVA), respectively. Linear regression was used to evaluate data scatter trends.

Patch pipette perfusion

Perfusion of patch pipettes made it possible to record activity from the same patch in the absence and presence of curare. The method we developed is based on a method described by Neher and Eckert (1988). A standard axial wire patch electrode holder (PC-S3, E. W. Wright, Guilford, CT) was modified by adding a port for the insertion a fine Teflon tube (Chemplast, Wayne, NJ), heat-stretched to a thinness of 20 μm i.d. at its tip, through which solutions could be added into the patch electrode. The tube was fastened along the length of the axial wire with Q-dope (G. C. Electronics, Rockford, IL) to provide support.

Patch electrodes (fabricated as described above) were prefilled with a small volume of control saline, filling up only the tapered portion of the tip. The perfusion tube on the holder was filled with a saline solution containing 20 μM curare. To prevent premature leakage of the contents of the perfusion tube into the patch pipette, some control solution from the pipette was drawn into the perfusion tube. Only patches with rapidly forming gigaseals were used, to reduce the possibility of curare being drawn into the pipette during seal formation. After ~2 min of recording with the control solution, air pressure was used for ~2 min, to drive the curare solution from the perfusion tube into the lumen of the patch electrode. The volume of solution in the pipette was increased by at least 10-fold during this procedure.

Solution mixing was generally complete 4 min after the initiation of perfusion, as judged by a higher steady level of channel activity. In data from nine patches analyzed manually, there was no increase in channel opening frequency, or evidence of any subconductance states before pipette perfusion, indicating that there was no significant pre-mixing of control and test solutions.

PARTIAL BLOCK MODEL

The experiments presented here will be discussed in terms of a model in which curare binds to a site in the channel pore. This binding is assumed to interfere with ion permeation resulting in a lower conductance of the open channel. The location of this "subconductance site" leads to specific predictions about the binding process, and thus about the kinetics of entering and leaving the subconductance state.

Curare is a large divalent cation, which is bigger than the largest molecules known to pass through the AChR channel (Huang et al., 1978; Dwyer et al., 1980). While one would not expect curare to pass all the way through the channel, it might enter to some extent. If curare bound to a site within the channel that lay within the transmembrane electrical field, then this binding would show voltage dependence. Hyperpolarization of the membrane would then favor the bound state. Furthermore, increasing the concentration of curare would increase the rate of binding to this putative binding site. This simple notion can be formulated as follows

$$\text{curare} + \text{AChR}_{\text{open-full}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{curare-AChR}_{\text{open-sub}}. \quad (3)$$

Here $\text{AChR}_{\text{open-full}}$ represents the receptor in its open conformation with the subconductance site unoccupied; $\text{AChR}_{\text{open-sub}}$ represents the open channel with its subconductance site occupied by curare, such that the conductance is reduced to the subconductance level. k_1 and k_{-1} represent the voltage dependent rate constants of curare binding and unbinding with this site. The above formula-

tion in terms of binding to the open AChR channel is not intended to exclude the possibility of binding to the subconductance site when the channel is closed. In fact, records indicate that this occurs about as often as binding when the channel is open (data not shown).

According to this model, the velocity of curare binding to the subconductance site V_1 (s^{-1}), can be determined by counting the number of transitions from the full conductance state to the subconductance state per unit of time spent in the full conductance state. This velocity divided by the curare concentration gives the rate constant for curare binding to the subconductance site k_1, ($M^{-1}s^{-1}$).

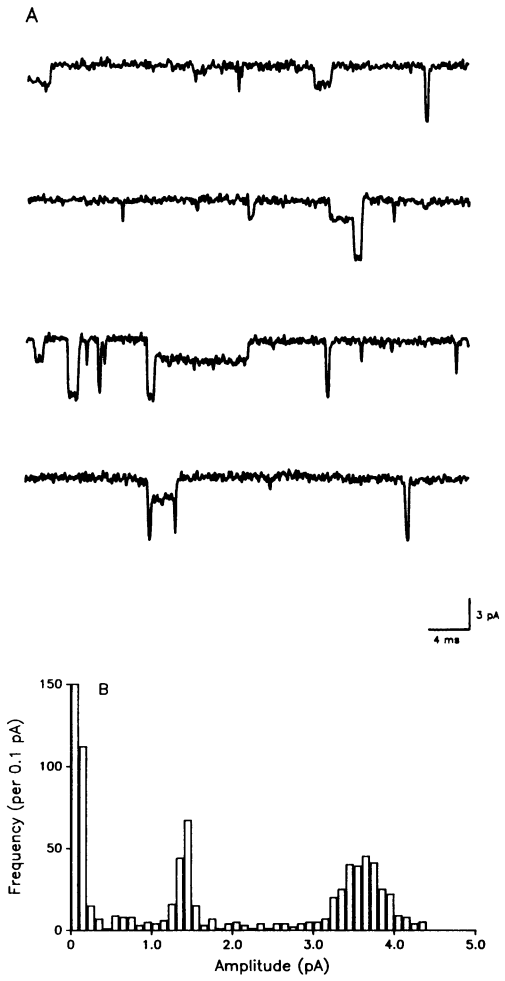

FIGURE 1 The subconductance state. (*A*) Single-channel currents from a patch exposed to 50 μM curare, membrane potential -135 mV, 3 kHz bandwidth. The subconductance state produces currents which are 39% of the full current amplitude. These subconductance states can appear either in isolation, immediately preceding, or immediately succeeding the fully open state. (*B*) Amplitude histogram of channel current openings from a different patch in the presence 100 μM curare. The membrane potential was held at -95 mV, resulting in smaller channel currents than in *A*.

The velocity of curare dissociation from the subconductance site, V_{-1}, is a unimolecular rate constant, k_{-1} (s^{-1}), which can be determined by counting transitions from the subconductance state to the full conductance state per unit of time in the subconductance state. The equilibrium dissociation constant, K_{eq}, is thus k_{-1}/k_1. We will examine how several experimental manipulations influence these parameters. In this way we will test various predictions of this model and characterize the putative subconductance site.

RESULTS

Single-channel currents in 50 μM curare are shown in Fig. 1 *A*. The patch electrode was clamped at +75 mV ($V_m \sim -135$ mV, see Methods). Assuming a linear current-voltage relation with 0 mV reversal potential, the full conductance state exhibits a conductance of 39 pS, while the subconductance state is 15 pS. Roughly 8.6 ± 1.4% (±SEM) of all full conductance openings are associated with a subconductance event at this concentration and voltage. This value was independent of the widely varying frequency of channel openings in different patches, indicating that the subconductance state transitions do not reflect the chance superposition of independent channel openings of different amplitudes. Fig. 1 *B* is an amplitude distribution from a different patch in the presence of 100 μM curare ($V_m = -95$ mV). Despite the lower amplitudes due to the lower V_m, the sub and full conductance states are clearly resolved.

In the course of analysis of thousands of spontaneous openings of the AChR channel (in the absence of agonist) for this and previous publications (Jackson, 1984; 1986), we saw no clear subconductance events. In the presence of CCh, subconductance states were exceedingly rare. As noted above, in the presence of curare subconductance events occur with a high frequency. Thus, the rate of the transition from the full to the subconductance state depends on ligand.

Effect of voltage and curare concentration on subconductance state kinetics

Voltage dependence

The influence of membrane potential on the rate of exit from the subconductance state (k_{-1}), is shown in a semilogarithmic plot in Fig. 2 *A*. The straight line is the least squares fit of the logarithm of k_{-1} to V_m. Rates determined at various concentrations are plotted together, as this unbinding rate is not dependent on the concentration of curare (see Fig. 3). k_{-1} increased e-fold

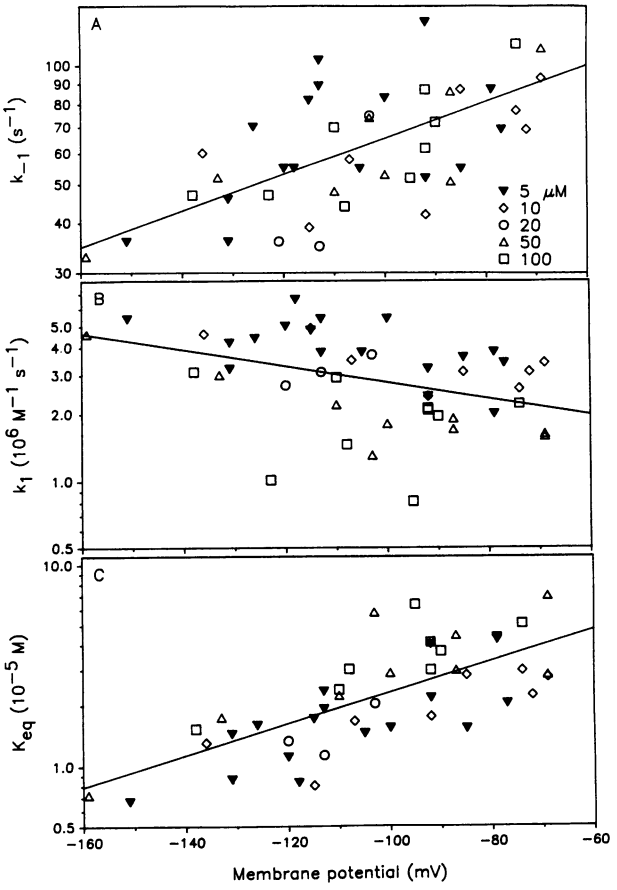

FIGURE 2 Semilogarithmic plots showing the effect of membrane potential on the kinetics of curare-induced subconductance states. (*A*) Effect of potential on the unbinding rate (k_{-1}) of curare from the putative subconductance site. The regression line ($r = 0.628$, $n = 46$, $p < 0.01$, 2-tail) indicates that as the membrane is depolarized, k_{-1} increases e-fold per 95 mV. (*B*) Effect of potential on the binding rate constant (k_1) of curare to the subconductance site. The regression line ($r = 0.407$, $n = 46$, $p < 0.01$, 2-tail) indicates that depolarization decreases k_1 e-fold per 118 mV. (*C*) Effect of potential on the equilibrium dissociation constant K_{eq}. Regression indicates an e-fold increase per 55 mV depolarization ($r = 0.680$, $n = 46$, $P < 0.01$, two-tail).

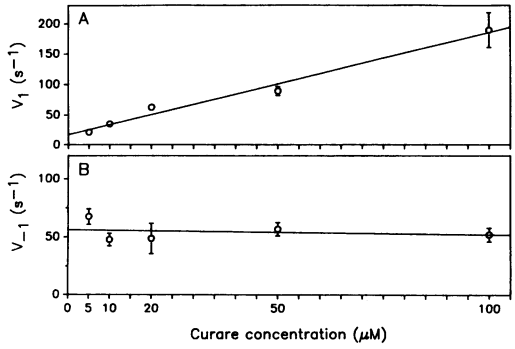

FIGURE 3 Effect of curare concentration on the velocity of channel entry into ($V_1 = k_1 \cdot$ mol curare) and exit from ($V_{-1} = k_{-1}$) the subconductance state. These data are from the same experiments used to make Figs. 2, *A* and *B*, but include only those values taken within a narrow range of voltages, to minimize the demonstrated effect of membrane potential on these values (see text and Fig. 2). (*A*) Effect of curare concentration on the velocity of entry into the subconductance state. As curare concentration increases, V_1 increases ($p < 0.0002$, ANOVA) in a linear fashion. The number of experiments at various concentrations of curare are 11 in 5 μM, 4 in 10 μM, 3 in 20 μM, 5 in 50 μM, and 6 in 100 μM. (*B*) Effect of curare concentration on the velocity of exit from the subconductance state. As curare concentration is increased, V_{-1} does not change significantly ($p < 0.33$, ANOVA). The number of experiments at various concentrations of curare are 10 in 5 μM, 3 in 10 μM, 3 in 20 μM, 4 in 50 μM, and 4 in 100 μM. Error bars are standard errors of the mean and the best fitting lines are drawn. The number of experiments plotted in *A* and *B* are different due to the use of slightly different voltage ranges for averaging V_1 and V_{-1} (see text).

per 95 mV depolarization. Likewise, Fig. 2 *B* shows that the binding rate, k_1, decreases e-fold per 118 mV depolarization. The apparent dissociation constant for this process, $K_{eq} = k_{-1}/k_1$, is plotted in semilog fashion as a function of voltage in Fig. 2 *C*. At 0 mV, $K_{eq} = 14.0 \times 10^{-5}$ M, and increases e-fold per 55 ± 9 mV, as calculated from the slope and the S.D. of the regression line. Hyperpolarization clearly favors the subconductance state in a manner consistent with our hypothesis invoking a subconductance site within the transmembrane electric field.

The approach of Woodhull (1973), originally applied to proton block of sodium channels, has been adapted to local anesthetic block of AChR (Neher and Steinbach, 1978), and is applicable to the model being examined here for the subconductance state. The voltage dependence of the reaction can be expressed as,

$$K_{eq}(V) = K_{eq0}e^{\delta zFV/RT}, \quad (4)$$

where V represents the membrane potential, K_{eq0} is the equilibrium dissociation constant when $V = 0$, δ is the fraction of the transmembrane potential traversed at the binding site within the channel, F is Faraday's constant and z is the valence of the blocking agent. Curare is a divalent cation, and the distance between its two charges is considerable, so an assumption must be made about whether one or both charges enter the membrane field. Assuming $z = +1$ gives $\delta = 0.46 \pm 0.07$, and assuming $z = +2$ gives $\delta = 0.23 \pm 0.04$ (±S.D.)

Concentration dependence

If the subconductance events are allosteric transitions of the curare liganded AChR channel, then we might expect the rate of transition from the full conductance state to the subconductance state to have little if any concentration dependence. On the other hand, if the subconduc-

tance state results from curare binding to a site other than the two agonist binding sites, then increasing the concentration of curare will increase the rate of partial block.

Figs. 3, *A* and *B* show the effect of curare concentration on the velocities of the hypothetical blocking (V_1) and unblocking (V_{-1}) reactions. The data plotted are from the same experiments as in Figs. 2, *A* and *B*, taken from recordings within the membrane potential range of −80 to −120 mV for V_1, and −100 to −140 mV for V_{-1}. Using these narrow ranges minimizes the effects of voltage. The velocity of the blocking rate is seen to increase nearly linearly with concentration (Fig. 3 *A*), suggesting first order binding of curare to a site on the AChR channel. The velocity of the unblocking reaction is independent of curare concentration (Fig. 3 *B*), which is expected for a dissociation process of the type described in Eq. 3 above.

Effect of curare on spontaneously opening channels

If ligand induces subconductance states by partial block of the channel, then a channel that has opened spontaneously might be as susceptible to this block as a channel that was opened by curare. By treating the agonist binding sites of the AChR with DTT and NEM we were able to produce receptors which were much less readily activated by curare, but nevertheless opened spontaneously. A few receptors escaped modification, so we utilized pipette perfusion, whereby the same patch can be recorded from in the absence and in the presence of curare. We recorded channel activity in a patch before the addition of curare (spontaneous activity), and compared this with channel activity after curare was added and assumed that this constituted a sum of spontaneous plus curare-induced activity. Thus we estimated the fraction of channel openings, which were due to spontaneously opening channels in the presence of curare. Next, we compared the relative rates of subconductance state occurrence in patches in which spontaneous openings comprised either a low proportion (control cells) or a high proportion (DTT-NEM treated cells) of openings.

Fig. 4 shows chart pen records of single-channel activity in patches from control (untreated), and DTT-NEM treated cells. These recordings show channel activity in the absence of curare (before time 0, purely spontaneous openings), during the perfusion of curare into the electrode (0 to 2 min), and after complete mixing of the curare and control pipette solutions (after 4 min, spontaneous plus curare-activated openings). Thus in patches from control cells, only a small proportion of openings in the presence of curare is spontaneous, while in the treated cells, the majority of the channel openings are spontaneous when curare is present. Fig. 5 summarizes the results of these experiments. The ratio of the total number of channel events occurring during the 2-min period preceding curare perfusion, to the total number of channel events during the 2 min period after curare mixing was complete, gives the fraction of spontaneous events occurring in the presence of curare (Fig. 5 *A*). The fraction of spontaneous events in the presence of curare was higher in DTT-NEM treated cells than in the control cells ($P < 0.03$, T-test, one-tail), presumably because the

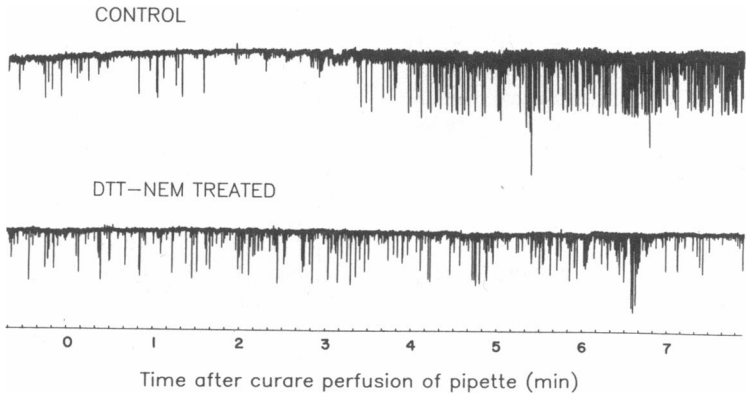

FIGURE 4 Chart pen records of pipette perfusion experiments. At time 0 min the recording pipette, which contains only a small volume of control saline, is perfused with a much larger volume of solution containing 20 μM curare. At 2 min, the perfusion is stopped and mixing of the recently introduced curare continues. Most of the single-channel activity before 2 min represents channels that are opening spontaneously before curare reaches the patch. After 4 min the mixing of curare in the pipette is essentially complete, and a higher level of channel activity, reflecting curare-activated channels as well as spontaneously opening channels, is reached. (*A*) Curare perfusion on a control patch produces a dramatic increase in channel activity, as curare activates the channels. (*B*) In contrast, it is seen that curare perfusion on a DTT-NEM treated patch results in only a slight increase in activity, indicating that curare can activate very few channels in the treated patch.

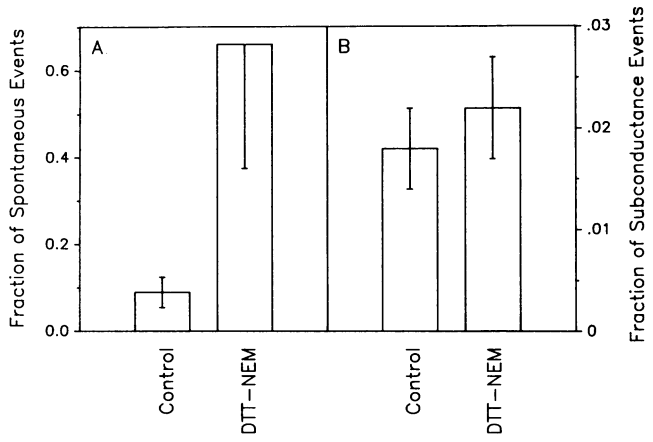

FIGURE 5 Effect of DTT-NEM treatment on the fraction of spontaneous and subconductance channel openings in the presence of curare. (*A*) The fraction of spontaneous events is compared in control vs. DTT-NEM treated cells. This fraction was determined from the increase in channel activity resulting from the perfusion of curare into the recording pipette (see Fig. 4 and text). The fraction of spontaneously opening channels in the presence of curare, is higher ($P < 0.03$, *T* test, one-tail) in DTT-NEM treated cells (0.66 ± 0.29, $n = 4$), than in control cells (0.09 ± 0.04, $n = 5$; mean ± SEM). (*B*) The fraction of subconductance events (see text) is compared in DTT-NEM treated versus control cells, in the same experiments as in *A*. The fraction of subconductance events in DTT-NEM treated cells is indistinguishable from that in control cells (0.022 ± 0.005 vs. 0.018 ± 0.004, respectively, $P < 0.6$, *T* test, two-tail).

chemical modification of the receptors prevented their activation by curare.

If curare-induced subconductance events are independent of the agonist activity of curare, then patches showing a much higher proportion of spontaneous openings (as after DTT-NEM treatment) should show the same level of subconductance activity as patches in which spontaneous openings represent a smaller fraction of all openings (as from untreated cells). Fig. 5 *B* shows the ratio of the number of subconductance events to the number of subconductance plus full conductance events in DTT-NEM treated and control cells. The same data were used to yield the values plotted in both Fig. 5, *A* and *B*. The relative frequency of the subconductance state does not differ between control and DTT-NEM treated cells ($P < 0.58$, T-test, two-tail). This relative frequency measure is sensitive to the mean open time of full conductance openings. In order for the above comparison to be meaningful, the average open time (ratio of the total time spent in the full conductance state to the number of full conductance events) of full conductance openings should not differ significantly between DTT-NEM treated and control cells. There were no significant differences in average full conductance open times between DTT-NEM treated versus control cells either before the addition of curare (0.77 ± 0.24 ms vs. 0.78 ± 0.38 ms, $P < 0.5$) or after curare (0.89 ± 0.18 vs. 0.71 ± 0.08, $P < 0.15$; mean ± SEM, T-test, two-tail). Thus the likelihood of a subconductance state occurring depends only on the presence of curare, and is the same whether the channel opens spontaneously or is activated by agonist. This result advances the argument that curare induces the subconductance state by action at a site other than the agonist binding sites.

Subconductance states in mixtures of curare and CCh

As an additional test of whether the agonist binding sites of the AChR are involved in subconductances, we used mixtures of CCh and curare to alter the state of occupancy of the agonist binding sites. If there is a subconductance site distinct from the two agonist binding sites, occupation of agonist binding sites by CCh instead of curare should have no effect on curare-induced subconductance events. CCh alone produces a negligible rate of subconductance events in this preparation (data not shown). We recorded from patches exposed to 5 μM curare alone, or mixed with 10, 20, 50, or 100 μM CCh, and compared the rates of occurrence of subconductance events.

Open time distributions of the full conductance state

It is important in these experiments with mixtures to know whether CCh is indeed occupying the agonist binding sites. An estimate of the degree to which CCh excludes curare from these sites can be made, based on the different durations of the full conductance state of channels gated by CCh or curare. Fig. 6 shows examples of open time distributions and superimposed theoretical fits. Fig. 6 *A* is the distribution in the presence of 5 μM curare alone, and Fig. 6 *B* is the distribution in the presence of 5 μM curare mixed with 100 μM CCh. Maximum likelihood analysis indicated that distributions of open times, with or without added CCh, were best described by a sum of two exponentials. The parameters obtained from curve fitting are the time constants τ_{sh} and τ_{lo} (reciprocal of the decay constants α_i from Eq. 1) which represent the short and long duration components of the distribution respectively, and x_{sh} and x_{lo} which represent their respective weightings. The average values of τ_{sh} and τ_{lo} vs. the concentration of CCh mixed with 5 μM curare are shown in Fig. 7. τ_{sh} (Fig. 7 *A*) and τ_{lo} (Fig. 7 *B*) both increase with the concentration of CCh ($P < 0.005$ and $P < 0.0001$, respectively, ANOVA). The ratio of the fraction of long to short duration channel openings, x_{lo}/x_{sh}, is also plotted vs. the concentration of CCh (Fig. 7 *C*), and this too increases ($P < 0.0001$, ANOVA).

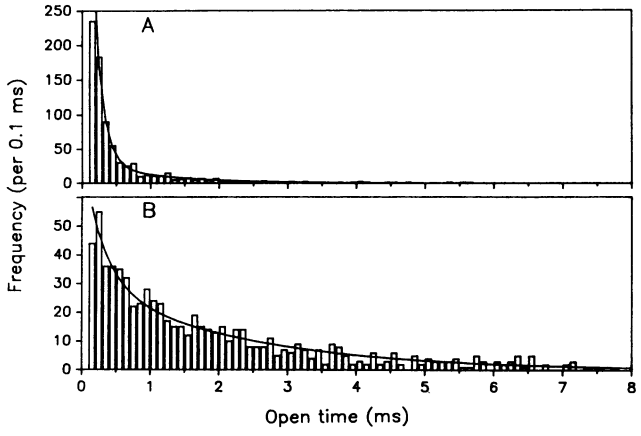

FIGURE 6 Lifetime histograms of the fully open state in CCh-curare mixtures. (*A*) Open time density from a patch exposed to 5 μM curare alone. The superimposed theoretical fit contains two exponential components with time constants τ_{sh} = 0.13 ms, and τ_{lo} = 1.0 ms, with the latter accounting for 20% of all openings (x_{lo} = 0.2). (*B*) Open time distribution from a patch exposed to a mixture of 5 μM curare plus 100 μM CCh. The time constants are τ_{sh} = 0.3 ms, and τ_{lo} = 2.2 ms, with the latter accounting for 83% of all openings.

These open time distributions of full conductance openings can be used to make some inferences concerning the occupancy of the agonist binding sites. In the case of open-time distributions with only one agonist present, there is a clear correspondence between the state of receptor occupancy and the relative densities of two exponential components. Long duration channel openings result from doubly liganded receptors and short duration channel openings result from singly liganded receptors (Colquhoun and Sakmann, 1985; Jackson, 1988). In the simplest case, with high concentrations of CCh alone, a single exponential with a time constant of from 4 to 6 ms reflects the dominance of channels gated by the binding of two CCh molecules. (Jackson, 1988). Our data from mixtures of CCh and curare exhibit a general increase in the open time with increasing CCh. However, it is not possible to analyze these data in complete detail because the many possible combinations of binding site occupancy introduce too many kinetic species. These states are not revealed in open time distributions, which are well described by a sum of only two exponentials.

Despite these complexities, open times for these mixtures become more characteristic of CCh gating at high concentrations of CCh. The τ_{lo} value of 2.6 ms in 5 μM curare plus 100 μM CCh, approaches a value characteristic of channels gated by two molecules of CCh. These long duration openings account for 77% of all openings. The discrepancy between this value of τ_{lo} and the value observed with CCh alone (4–6 ms), can be explained by invoking open channel block by curare (Marty et al., 1976; Manalis, 1977; Katz and Miledi, 1978; Colquhoun et al., 1979; Takeda and Trautmann, 1984), and by CCh (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985). Longer duration openings, such as those produced by two CCh molecules, would be particularly susceptible to this process. Therefore, analysis of open time distributions indicates that in 5 μM curare plus 100 μM CCh, a large fraction of openings (77%), and a larger fraction of the total open time (96%) is accounted for by receptors in which both agonist binding sites are occupied by CCh.

Subconductance state kinetics

The velocities of channels entering and leaving the subconductance state in CCh mixtures with 5 μM curare are plotted vs. CCh concentration in Fig. 8. Despite the fact that CCh-gated channels are producing most of the total open time in high CCh mixtures (we estimate 96% with 100 μM CCh plus 5 μM curare), the velocity of entry to the subconductance state, V_1, in 5 μM curare is reduced by only 38% by the addition of 100 μM CCh. V_1 is not affected at all when CCh is 50 μM or less (Fig. 8 *A*). If curare elicits subconductance events by binding to the

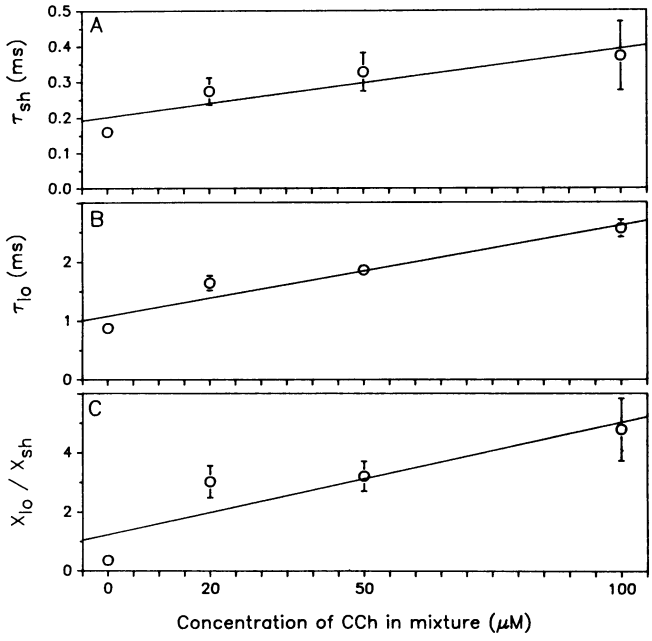

FIGURE 7 Variation of lifetime distributions of the fully open state with CCh concentration in CCh-curare mixtures. The curare concentration was 5 μM in all of these mixtures. The parameters were determined by maximum likelihood fits of a sum of two exponentials (Eq. 1) to data such as that used to construct the open time histograms in Fig. 6. (*A*) As CCh is increases, τ_{sh}, the short time constant, increases ($p < 0.005$, ANOVA). (*B*) τ_{lo}, the long time constant, and (*C*) x_{lo}/x_{sh}, the relative abundance of long duration to short duration openings, also increase with CCh. The number of experiments were 18 in 5 μM curare alone (CCh = 0), 8 in 20 μM CCh + 5 μM curare, 12 in 50 μM CCh + 5 μM curare, and 7 in 100 μM CCh + 5 μM curare.

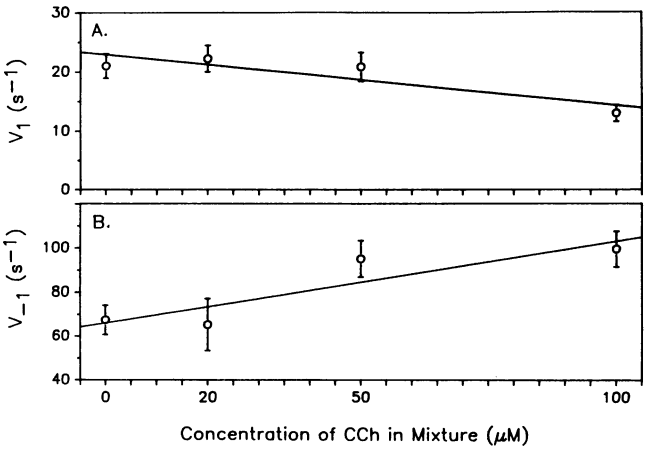

FIGURE 8 The velocity of curare entry to the subconductance site, V_1, and exit from this site, V_{-1}, in mixtures of 5 μM curare plus various CCh concentrations. (*A*) As the concentration of CCh is increased up to 50 μM, V_1 does not change. When CCh = 100 μM in the mixture, V_1 falls to 62% of its value at lower CCh concentrations. The number of experiments at the various CCh concentrations (plus 5 μM curare) are 11 in 0 μM CCh, 9 in 20 μM CCh, 6 in 50 μM CCh, and 9 in 100 μM CCh. (*B*) The velocity of the unblocking reaction V_{-1} is influenced by CCh as well ($p < 0.021$, ANOVA). The number of experiments at the various CCh concentrations (plus 5 μM curare) are 10 in 0 μM CCh, 9 in 20 μM CCh, 6 in 50 μM CCh, and 8 in 100 μM CCh. The number of experiments plotted in *A* and *B* are different due to the use of slightly different voltage ranges for averaging V_1 and V_{-1} (see text).

agonist binding sites, then the occupation of these sites by CCh for 96% of the open time would result in a comparable reduction in V_1. Fig. 8 *B* shows that the rate of leaving the subconductance state is also affected by the presence of CCh ($P < 0.021$, ANOVA). Thus it appears that occupation of agonist binding sites by CCh does not prevent the generation of subconductance states by curare, but it does slightly inhibit them.

DISCUSSION

The results presented here are consistent with the hypothesis that curare-induced subconductance states are the result of binding to a distinct subconductance site of the AChR that lies within the transmembrane electric field. This hypothesis was also favored by Takeda and Trautmann (1984). Various aspects of this model are supported while alternative models are challenged by our findings.

Agonist binding sites are not involved

The alternative model most clearly challenged by our results is that in which curare induces the subconductance state as an allosteric transition upon binding to the agonist binding sites. It is difficult to reconcile such a picture with the results presented here. The capacity of curare to induce the subconductance state is diminished only slightly or not at all by manipulations of agonist binding sites that should exclude curare.

Modification of the agonist binding sites by DTT-NEM treatment clearly reduces agonist activation of the receptor (Karlin and Bartels, 1966; Karlin, 1980; Walker et al., 1981; Jackson, 1984). There is some debate whether this treatment modifies one or both of the agonist binding sites of the AChR (Froehner et al., 1977; Walker et al., 1981; de Souza Otero and Hamilton, 1985). It is also not clear whether this modification prevents agonist binding or disrupts the translation of agonist binding to channel opening (Damle and Karlin, 1980). Although the small increase in channel opening after curare perfusion onto patches in DTT-NEM treated cells suggests that most openings in the presence of curare are still spontaneous, the possibility that curare still binds to the agonist binding sites in treated cells cannot be ruled out. DTT-NEM treatment does not, however, change the ability of curare to induce subconductance events. A model in which transitions to the subconductance state are induced allosterically must address the question of how DTT-NEM treatment results in a major decrease in the rate of channel opening, but does not alter the equilibrium between the full conductance and the subconductance states. The partial block model of curare-induced subconductances provides a simple explanation for this result.

Experiments with CCh-curare mixtures strengthen the case for a distinct subconductance site. As the CCh concentration was increased, while keeping the curare concentration constant, the open time distribution became dominated by longer duration openings. Comparisons with the open time distributions produced by high concentrations of CCh alone (Jackson, 1988) indicate that the long duration openings in these mixture experiments are likely to be openings of channels in which both agonist binding sites are occupied by CCh. With a 5 μM curare plus 100 μM CCh mixture, these openings account for 96% of the total time in the open state. In the absence of curare, CCh-gated channels enter subconductance states very rarely. If occupation of an agonist binding site by curare were necessary for curare to induce a subconductance state, the displacement of curare from these sites by CCh would alter the subconductance state kinetics in proportion to the degree of curare displacement. The result of this experiment suggests that curare-induced subconductance states do not depend on curare binding to the agonist binding sites.

Clearly, the subconductance state is dependent on the presence of a ligand, since it is not seen when ligand is absent, and is seen rarely in the presence of CCh alone. By separating the role of the agonist binding sites from

subconductance state generation, we have been able to conduct experiments that support the idea of a separate site to which curare can bind to reduce the channel conductance of the AChR. The linear dependence of the rate of subconductance state generation on curare concentration suggests that each receptor has one such subconductance site. This site has an apparent dissociation constant of 140 μM at a membrane potential of 0 mV.

Increasing the CCh concentration form 0 to 100 μM in the presence of 5 μM curare reduces the rate of entry to the subconductance state by 38% (Fig. 8 *A*). This change is small but significant. A weak but significant effect of CCh concentration on the rate of exit from the subconductance state was also noted. However, there is too much subconductance activity in 100 μM CCh for us to reject the hypothesis involving a separate subconductance site. A more likely explanation is that there is a weak negative interaction between one or both of the agonist binding sites and the subconductance site. This interaction could be a Coulombic repulsion between the ligands, if the agonist binding and the subconductance sites are not far apart. Consistent with this interpretation is the non-zero intercept of the plot of the apparent velocity of binding of curare to the subconductance site versus curare concentration (Fig. 3 *A*). Though not plotted as such, zero is a real experimental point; there are no subconductance events in spontaneous openings in the absence of ligand. Thus the velocity of channel entry into the subconductance state appears to increase more slowly above a curare concentration of 5 μM. As suggested above, this may be due to occupation of the agonist binding sites at higher curare concentration, resulting in electrostatic repulsion between the agonist binding and subconductance sites. However, the slightly higher velocity of subconductance state exit at 5 μM curare (Fig. 3 *B*) is not easily explained by this model. The clarification of these subtle effects will require additional experiments.

Partial block of the channel

While a strong case can be made for the existence of a distinct subconductance site, the main reason we have for suspecting that this site is within the ion permeation pathway is the voltage dependence of the subconductance state kinetics. However, this is based on the assumption that the subconductance site does not change conformation with voltage. Hyperpolarization favors the subconductance state. A simple explanation for this is that the subconductance site is within the pore and is thus influenced by the transmembrane electric field. The fraction of the membrane potential traversed by a curare molecule upon binding to this site is 0.46 or 0.23 depending upon whether one counts one or both positive charges of curare. It appears that the voltage dependence of the binding rate, k_1, is weaker than the voltage dependence of the unbinding rate, k_{-1}. This might be due to a diffusion limitation on the frequency of collisions of curare with the subconductance site and a small energy barrier to the binding step. Diffusion limited rates for ligand-binding processes are on the order of 10^8 M^{-1} s^{-1} (Fersht, 1985). This value is 30-fold higher than our k_1. Some of this difference might be accounted for by the large size and the divalent charge of curare. Thus, diffusion might limit the frequency of collisions, while voltage might influence the fraction of collisions which are productive and result in binding.

Channel block models are often used to explain the actions of substances at the AChR (Adams, 1976; Neher and Steinbach, 1978; Adams and Sakmann, 1978; Ogden and Colquhoun, 1985), and at other channels (Armstrong and Binstock, 1965; Woodhull, 1973). Indeed, curare can block open channels completely (Manalis, 1977; Colquhoun et al., 1979). The estimates made for the fraction of the membrane potential traversed in the latter case were 0.80 or 0.40, depending on whether one or two charges of curare contribute to this voltage dependence. This larger fraction for full block as compared with partial block may reflect a deeper penetration of curare into the channel when total block is effected. Total block of the channel by curare is suggested by the decrease in the mean channel open time with increasing curare concentration (Trautmann, 1982). QX-222, which blocks the channel completely, traverses nearly 80% of the transmembrane potential (Neher and Steinbach, 1978). Thus there may be more than one site to which curare and other channel blockers can bind within the channel. The two rings of negative charge at the extracellular side of the channel vestibule (Imoto et al., 1988) could provide a varied surface with several possible sites to attract the positive charges of curare and other blockers.

Interestingly, Takeda and Trautmann (1984), found that the full conductance states induced by ACh or curare appear susceptible to complete block by curare, but the curare-induced subconductance state is not, since the mean duration of this state is not reduced by curare. This suggests that in occupying the subconductance site, curare restricts the access of pore blocking agents to the site associated with total channel block. The apparent proximity of these two blocking sites lends further support for the independence of the subconductance and the agonist binding sites.

It is notable that total block of the AChR channel by QX-222 at low concentrations occurs almost exclusively when the channel is open (Neher and Steinbach, 1978). In contrast, when curare is present AChR channels can enter a subconductance state from either the closed or open state. Thus, it appears that the subconductance site

differs from the local anaesthetic binding site in that the former is accessible regardless of whether the channel is open or closed. The question of how the rates of association and dissociation to the subconductance site depend on the conformation of the channel is beyond the scope of the present study.

While partial block of the channel pore by curare paints a simple and consistent picture, we cannot rule out other models in which the subconductance site is distant from both the channel pore and the agonist binding sites. The occupation of such a site could induce an allosteric transition to the subconductance state. A movement of charge during this conformational change could then account for the observed voltage dependence. In this context it is worth noting that the voltage dependence of the mean open time of ACh-gated channels (Neher and Stevens, 1979), and of the rate of spontaneous opening (Jackson, 1986), indicates that some charge movement does occur during channel gating.

CONCLUSION

Subconductance states are a common feature of many different channels, and are generally assumed to arise from different conformations of the open channel (Hamill et al., 1983; Morris and Montpetit, 1985; Jahr and Stevens, 1987). Our findings that the curare-induced subconductance state is consistent with a partial blocking mechanism raises the possibility that some of the subconductance states observed for other channels might also arise form a partial block rather than alternative open conformations.

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