AGONISTS BLOCK CURRENTS THROUGH ACETYLCHOLINE RECEPTOR CHANNELS

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ABSTRACT We have examined the effects of high concentrations of cholinergic agonists on currents through single acetylcholine receptor (AChR) channels on clonal BC3H1 cells. We find that raised concentrations of acetylcholine (ACh; above 300 μM) or carbamylcholine (Carb; above 1,000 μM) produce a voltage- and concentration-dependent reduction in the mean single-channel current. Raised concentrations of suberyldicholine (Sub; above 3 μM) produce a voltage- and concentration-dependent increase in the number of brief duration low-conductance interruptions of open-channel currents. These observations can be quantitatively described by a model in which agonist molecules enter and transiently occlude the ion-channel of the AChR.

INTRODUCTION

At the vertebrate motor synapse nerve-released acetylcholine (ACh) activates receptors (AChRs) in the muscle membrane. ACh binds to two specific sites on the AChR, resulting in the opening of a cation-selective membrane channel formed by the AChR. Numerous organic cations block the ACh-induced permeability increase by binding to a site on the AChR that is distinct from the sites for ACh binding. For compounds such as local anesthetics this site appears to be located within the ion channel of the AChR.

The agonists that activate the AChR are organic cations and might be expected to interact with the cation-selective AChR channel (e.g., Adams et al., 1981). Indeed, decamethonium (Adams and Sakmann, 1978) and +-tubocurarine (Morris et al., 1982; Trautmann, 1982) have been reported to both activate AChRs and to block ion flow through the AChR channel. We have found that all of the agonists examined, including acetylcholine, cause transient blocking of the AChR channel. These observations were made in the course of experiments examining the dose-dependence of activation of AChRs using records of currents through single channels (see Sine and Steinbach, 1984). The presence of this additional interaction between agonists and the AChR complicates the design and interpretation of dose-response studies. In particular, channel blockade will affect both microscopic and macroscopic measurements of membrane current in biophysical studies and ion flux in biochemical studies of receptor function. A preliminary description of these findings has appeared (Sine and Steinbach, 1983).

METHODS

We studied the AChR found on a clonal mouse cell line, BC3H1 (Schubert et al., 1974). These cells express AChRs with properties of skeletal muscle nicotinic AChR (reviewed in Sine and Steinbach, 1984). BC3H1 cells were carried as described (Sine and Taylor, 1979). For biophysical studies cells were plated at low density on glass coverslips and maintained 10 to 14 D in Dulbecco's Modified Eagles medium plus 0.5% cadet calf serum (Sine and Steinbach, 1984). Currents through single channels were recorded using the giga-ohm seal patch-clamp technique with the "cell attached," "inside out," or "outside out" patch configurations (Hamill et al., 1981). The extracellular and intracellular solutions were symmetrical in K, Na, Cl, and HEPES. For experiments with outside-out patches, solutions had the following compositions: K, 140 mM; Na, 10 mM; Cl, 150 mM, HEPES, 25 mM (pH 7.4). The extracellular solutions also contained agonist, $CaCl_2$, 1.8 mM and $MgCl_2$, 1.7 mM. The intracellular solutions contained $MgCl_2$, 2.0 mM, and EGTA, 1 mM. Experiments with inside-out patches used 150 mM K, 0 Na, with other concentrations as listed above. The temperature was controlled by a Peltier cooling device and measured with a small probe placed in the solution near the coverslip.

Data were recorded on an FM tape recorder (Racal Store 4D, Sarasota, FL; 7½ or 15 in/s), then replayed and filtered for analysis. Data were filtered with an 8-pole Bessel filter (model 902LPF, Frequency Devices, Haverhill, MA; cutofff frequencies given as −3db point) and digitized and stored in digital form using a PDP 11/34 minicomputer (Digital Equipment Corp., Marlboro, MA). Channel opening and closing transitions were detected using a threshold set in the middle of the open channel deflection from baseline and durations of open and closed states were defined as the number of sample points between threshold crossings. A minimum acceptable duration was imposed during the analysis. For current-voltage curves data were filtered at 500-1,000 Hz, digitized at 100 to 200-μs intervals, and the mean current was measured for openings lasting longer than 0.5 to 1 ms. The data used for kinetic analysis of suberyldicholine action were filtered at 3,200 Hz, digitized at 50-μs intervals, and a minimum duration of 150 μs was imposed for both open and closed channel states.

RESULTS

The key observations are shown in Fig. 1, which illustrates the effects of raising the concentrations of acetylcholine (ACh) and suberyldicholine (Sub) on currents through AChR channels in the membrane of clonal BC3Hl cells. As the concentration of ACh is increased, the mean single channel current is reduced with an associated increase in the variance of the open channel current. As the concentration of Sub is increased, the open channel currents appear "ragged" as transient interruptions of the open channel current occur more frequently. These observations are qualitatively consistent with the idea that agonists transiently block ion flow through the open channel: Sub produces a relatively long-lived block analogous to block induced by local anesthetics (Neher and Steinbach, 1978), whereas ACh results in a very short-lived decrease in conductance, which cannot be resolved by these measurements.

To analyze the blocking action of ACh, the mean single-channel current was measured at several membrane potentials and ACh concentrations. The resulting current-voltage relationships are shown in Fig. 2. At low concentrations of ACh the current-voltage (I-V) relationship is linear, whereas at high concentrations the mean single-channel current clearly depends on ACh concentration and shows a nonlinear dependence on membrane potential. We

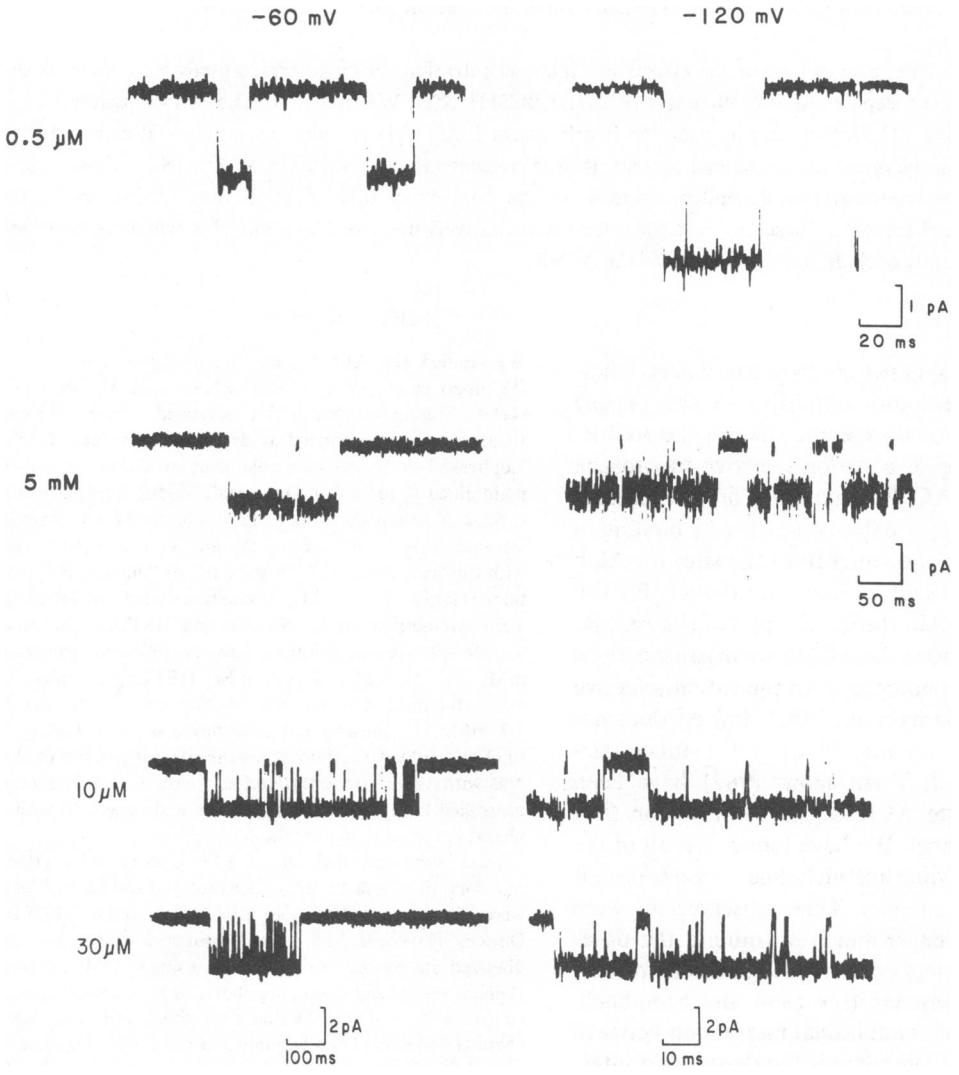

FIGURE 1 Oscilloscope records of currents through single AChR channels are shown at different concentrations of ACh (*top* four traces) and Sub (*bottom* four traces). Note that high concentrations of ACh decrease the mean current through the channel and increase the variance of the current through the open channel, especially at −120 mV. High concentrations of Sub (above 3 μM) cause many brief interruptions in the current, although the mean current is not reduced between interruptions. *Top* four traces: records of ACh induced currents shown from two inside-out patches at the concentrations and potentials specified, low-pass filtered at 1 KHz. *Bottom* four traces: records from one outside-out patch exposed to 10 and 30 μM Sub at one potential (−70 mV) shown at two sweep speeds (see scale bars). The concentration of Sub was established by exchanging the bath solution with nine volumes of the specified solution. *Left*: records low-pass filtered at 1 KHz; *Right*: records filtered at 3.2 KHz.

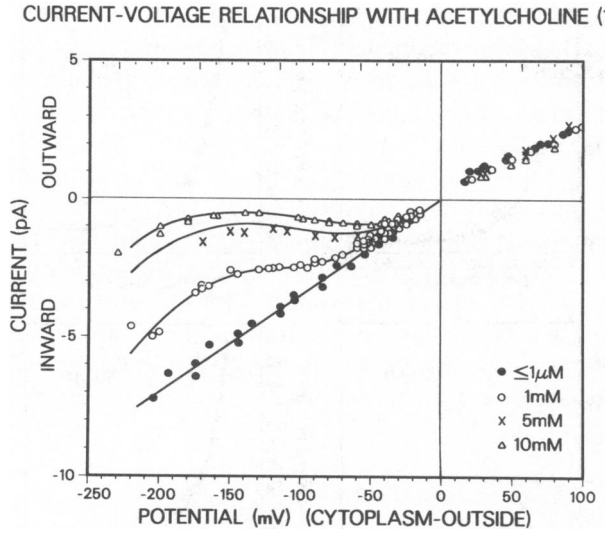

FIGURE 2 The mean current through single open channels is plotted at different applied membrane potentials and ACh concentrations (●, 1 μM; o, 1 mM; X, 5 mM; △, 10 mM). The lines were obtained by adjusting parameters in Eq. 1 (see text) to obtain the best fit by eye to all of the data shown. The parameters used in the figure (11°C) were $F(V) = 20\ M^{-1} \times \exp(-V \times 0.032\ mV^{-1})$; K_D at zero potential = 50 mM; $G(V) = 7.0 \times 10^{-4} \times \exp(-V \times 0.035\ mV^{-1})$. Similar data at 21°C with 1 μM, 1 mM, and 5 mM ACh gave values of $F(V) = 38\ M^{-1} \times \exp(-V \times 0.023\ mV^{-1})$; K_D at zero potential = 27 mM; $G(V) = 5.8 \times 10^{-4} \times \exp(-V \times 0.031\ mV^{-1})$. Less complete data for carbamylcholine (21°C; 1 μM, 1 mM, 3 mM carbamylcholine; potential range −30 mV to −100 mV) gave an estimate $F(V) = 9.8\ M^{-1} \times \exp(-V \times 0.032\ mV^{-1})$; K_D at zero potential = 110 mM. Each data point shows the mean current of 50 to 200 events from a given patch in the inside-out configuration in symmetrical 150 mM KCl solutions (see Methods). Identical data were obtained from cell attached patches. Similar results were obtained in 140 mM sodium salt solutions and when external Ca^{++} ions were completely replaced with Mg^{++} (not shown). 11°C. Records were low pass filtered at 500 Hz (−30 mV to +30 mV) or 1,000 Hz and digitized at 100- or 200-μs intervals.

have analyzed the blocking action of ACh in terms of a model in which ACh binds at a site within the channel that senses a portion of the voltage drop across the membrane (Woodhull, 1973)

$$A_E + R_o \underset{b(V)}{\overset{f(V)}{\rightleftharpoons}} R_b A \xrightarrow{p(V)} R_o + A_I.$$

Scheme I

External agonist (A_E) binds inside the channel of the active AChR (R_o) to form a nonconducting complex (R_b). The rate constants for binding, $f(V)$, and dissociation, $b(V)$, depend on membrane potential because the blocking site is located in the channel. Therefore, the equilibrium occupancy of the blocking site depends on both ACh concentration and membrane potential. The amplitude of single-channel currents increases at extreme hyperpolarizations, suggesting that ACh permeates the channel when sufficient driving force is applied. This relief from block is described by the reaction step with the voltage-dependent rate constant, $p(V)$, resulting in R_o and internal agonist (A_I, assumed to be negligible). Scheme I is used hereafter to interpret the results, and we consider alternative models in the Discussion.

We analyzed the I-V curves using Eq. 1, derived from Scheme I under equilibrium conditions. We have assumed that the rates depend exponentially on voltage, that $f(V)$ and $b(V)$ have equal and opposite voltage dependences (see data on Sub below), and that the current carried by ACh is negligible (since ACh is present at <5% of the concentration of other permeant ions). The I-V relationship is predicted to be

$$i'(V, A_E) = i(V)[1 + G(V)]/[1 + G(V) + A_E F(V)], \quad (1)$$

where $i'(V, A_E)$ is the mean current at a given potential and concentration of external ACh, and $i(V)$ is the mean current at that potential at a low concentration of ACh. The voltage-dependent functions, $F(V)$ and $G(V)$, were adjusted to fit the data. These functions are ratios of rate constants: $F(V) = f(V)/b(V)$ and, $G(V) = p(V)/b(V)$.

In Fig. 2 the continuous lines through the data were obtained by fitting all of the data simultaneously with the predictions from Scheme I. The parameters used in fitting this blocking scheme give the equilibrium dissociation constant for ACh occupation of the blocking site and the fraction of the membrane field sensed (see Woodhull, 1973). The estimated equilibrium dissociation constant is 50 mM at 11°C and 27 mM at 21°C for external ACh at zero potential, while the binding site is ~80% of the way through the membrane field (see Table I).

The reduction in block at extreme hyperpolarizations is marked as has also been observed for the block of squid axon potassium channels by sodium ions (French and Wells, 1977). When the entire I-V relationship is fit with Scheme I the high voltage sensitivity of the permeation step, $p(V)$, requires that the single charge on ACh senses more than the total applied field when it passes through the channel. This high voltage sensitivity may result from structural changes in the channel at extreme polarizations, reducing the internal barrier to ACh passage. Alternatively the AChR channel may be a multi-ion pore. In such a multi-ion pore, hyperpolarization would increase the occupancy of an ion-binding site external to the blocking site, which could cause an increase in the rate for ACh passage (see Hille and Schwarz, 1978, for a discussion of multi-ion pores).

Sub has an apparently different effect on currents through single AChR-channels than ACh does. As the concentration of Sub is raised, brief interruptions of the open channel currents occur more frequently (Fig. 1), but the current flowing between interruptions is not reduced. The blocking action of Sub, therefore, is apparent directly from analysis of open and closed durations. When Sub concentrations are varied between 10 and 30 μM the mean apparent open time decreases with increasing Sub concentration, whereas at a single concentration the mean open

time decreases with membrane hyperpolarization (Figs. 3 and 4).

The lifetime of the blocked state can be determined by analysis of the closed duration histograms. Short duration closed times, however, could reflect several processes in addition to channel block. In particular, brief closings could result from receptor activation processes. Fig. 3 shows that brief duration closed time histograms are described by the sum of two exponentials: a dominant fast component and a minor slow component. The major fast component apparently results from channel block because its amplitude is increased by conditions that increase block: increased concentrations of Sub and membrane hyperpolarization. Furthermore, there is no change in the mean duration of the fast component with increasing Sub concentration. Therefore, we consider only this major fast component of the closed duration histogram for analysis of the blocking action of Sub. The mean duration of blocking events, τ_b, does not change with Sub concentration but is prolonged when the membrane is hyperpolarized (Fig. 4).

The slow blocking action of Sub can be well described in terms of the same blocking model used to describe fast block by ACh, but with resoluble transition rates. The reciprocal of the open-time increases linearly with Sub concentration, and the calculated forward and backward rate constants depend exponentially on membrane potential over the range analyzed (Fig. 4). The forward and backward rate constants have equal and opposite voltage dependencies, resulting in more effective agonist block at hyperpolarized voltages. These voltage dependencies are consistent with the model in which one charge on the Sub molecule senses ~80% of the transmembrane field at the blocking site. The apparent K_D is 5.2 mM at 11°C and zero applied potential (Table I).

Analysis of preliminary data (not shown) obtained with Sub applied to cell-attached patches at potentials between −100 and −190 mV indicates that the mean open time decreases exponentially with hyperpolarization over this range, whereas the mean duration of the blocking events reaches a maximum at about −130 mV, but becomes shorter with further hyperpolarization. This reduction in

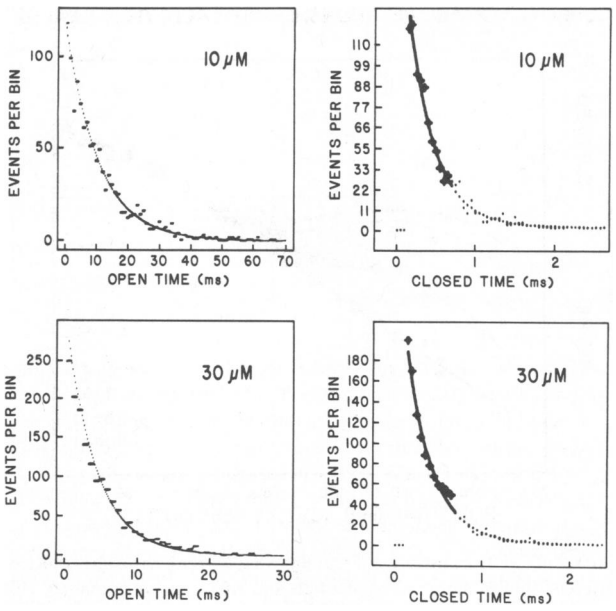

FIGURE 3 Histograms of open and closed durations from records of single channel currents elicited by 10 and 30 μM Sub applied to one outside-out patch. High resolution records (shown in Fig. 1, lower two traces) were low pass filtered at 3.2 KHz and digitized at intervals of 50 μs. Open and closed durations were detected that lasted 150 μs or longer using a detection threshold midway between the mean baseline and open channel currents (see Methods and Sine and Steinbach, 1984). The open time histograms were fitted with a single exponential using a nonlinear least-squares method. For the data shown: 10 μM Sub: τ_o = 10.1 ms, 1,174 events; 30 μM Sub: τ_o = 4.0 ms, 1,578 events. Closed duration histograms were fitted visually with the sum of two exponentials using a curve peeling technique. For the data shown: 10 μM Sub: τ_b = 306 μs; fraction = 0.87; τ_2 = 2.8 ms, fraction = 0.13. For 30 μM Sub: τ_b = 294 μs, fraction = 0.91; τ_2 = 1.6 ms, fraction = 0.09. τ_b was also estimated by subtracting the small contribution of τ_2 and fitting the resulting data with a single exponential using a nonlinear least-squares routine. This method gave similar estimates for τ_b of 328 and 287 μs for 10 and 30 μM, respectively. The major component, τ_b, is examined as a function of concentration and voltage in Fig. 4. We have not attempted to clarify the nature of τ_2 here, but suggest it may result from transitions between open and activatable receptor states. Note that open durations are briefer at higher Sub concentrations, whereas closed times do not change appreciably with Sub concentration.

TABLE I
PARAMETERS FOR CHANNEL BLOCK, RECEPTOR ACTIVATION, AND AGONIST BINDING

	$F(V)(M^{-1})$	K_D(0 mV)	K_{act}(0 mV)	K_p(inst)(0 mV)	K_p(equil)(0 mV)
ACh (21°)	38 exp $(-V \times 0.023\ mV^{-1})$	27 mM	—	—	—
ACh (11°)	20 exp $(-V \times 0.032\ mV^{-1})$	50 mM	—	—	—
Carb (21°)	9.8 exp $(-V \times 0.032\ mV^{-1})$	110 mM	71 μM	70 μM	18 μM
Sub (11°)	193 exp $(-V \times 0.032\ mV^{-1})$	5.2 mM	1.0 μM	0.96 μM	0.24 μM

The first column gives the ratio of rate constants for block ($f[V]/b[V]$; see text), the second column gives the calculated K_D for block at zero applied potential. The remaining columns give apparent K_D's determined in experiments using intact BC3H1 cells depolarized in K^+ Ringer's solution (Sine and Taylor, 1980, 1981; and S. M. Sine, unpublished observations). K_{act} is the concentration of agonist that stimulates 50% of the maximum initial rate of Na^+ influx. K_p(inst) and K_p(equil) are the concentrations of agonist that inhibit by 50% the initial rate of α-neurotoxin binding to the AChR. K_p(inst) is obtained under conditions of simultaneous addition of agonist and α-neurotoxin, and largely reflects agonist binding to resting and active states of the receptor. K_p(equil) is obtained after cells are pre-incubated with agonist, and reflects agonist binding to the equilibrium population of active and desensitized receptors at that agonist concentration.

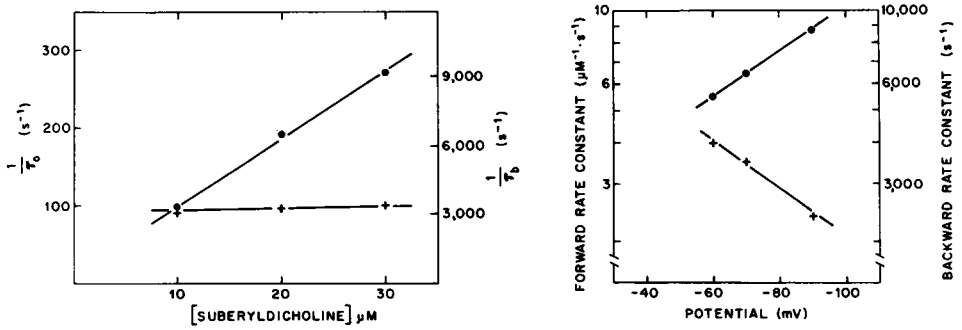

FIGURE 4 (*Left*) Concentration dependence of Sub forward blocking and backward unblocking rates. One outside-out patch was exposed to three concentrations of Sub at −70 mV. Histograms for open and closed durations were analyzed to give the mean open time (τ_o) and mean blocked time (τ_b) as described in the text and the legend to Fig. 3. (Histograms for 10 and 30 μM Sub are shown in Fig. 3.) Reciprocals of these times are plotted against the Sub concentration (τ_o^{-1} as forward rate, τ_b^{-1} as backward rate). τ_o^{-1} increases linearly with Sub concentration with a slope of 6.4 $\mu M^{-1} s^{-1}$, whereas τ_b^{-1} is essentially constant at 3,100 s^{-1}. In the linear blocking scheme, a plot of τ_o^{-1} against blocking molecule concentration is linear with a slope of $f(V)$, while τ_b^{-1} gives a value for $b(V)$. (*Right*) Dependence of Sub forward and backward rate constants on membrane potential. The forward blocking rate constant was determined from patches at single or multiple concentrations and potentials (3 to 7 patches per symbol). At each potential $f(V)$ was determined from the concentration dependence of the forward blocking rate as shown on the left panel (2 to 4 concentrations per potential). The backward rate constant, $b(V)$, was determined from the mean backward rate, τ_b^{-1}, measured at Sub concentrations between 10 and 30 μM (3 to 9 patches per potential). The lines shown give the following estimates: $f(V) = 2.2 \exp(-V \times 0.0153)$ $\mu M^{-1} s^{-1}$ and $b(V) = 1.14 \times 10^4 \exp(V \times 0.0173)$ s^{-1}.

Sub blocked time is consistent with the relief from block seen in the I-V relationships with high concentrations of ACh at extreme hyperpolarizations.

The forward blocking rates for ACh and Sub are of the same order of magnitude. Preliminary kinetic analysis of high resolution measurements using 300 μM ACh indicates the rate constant for leaving the site, $b(V) + p(V)$, is ~40,000 s^{-1} at −130 mV and 11°C. Therefore, the rate for entering the site, $f(V)$, is ~4.5 × 10^7 $M^{-1} s^{-1}$ at −130 mV. At −130 mV the forward rate for block by Sub is 1.6 × 10^7 $M^{-1} s^{-1}$. These estimates are fairly close to that for QX-222 acting on extrajunctional receptors on denervated frog muscle fibers (f = 2 × 10^7 $M^{-1} s^{-1}$ at −120 mV and 9°C; Neher and Steinbach, 1978).

Carbamylcholine shows effects on single-channel currents that are similar to those of ACh, although carbamylcholine has a 2–3-fold lower apparent affinity for the blocking site (Table I). Decamethonium at concentrations of 30 to 60 μM produces relatively slow block similar to the block elicited by high concentrations of Sub.

DISCUSSION

We have found that raised concentrations of agonists have concentration- and voltage-dependent effects on currents through AChR channels. These effects can be quantitatively and simply explained by the assumption that agonists transiently bind to sites inside the channel and block the flow of ions through the channel (Woodhull, 1973). When sufficient driving force is applied, agonist molecules can pass completely through the AChR channel producing relief of block. Previous work has shown that decamethonium produces changes in endplate current relaxations following voltage steps that are consistent with the idea that decamethonium is a channel-blocking compound (Adams and Sakmann, 1978), and it has been reported that radioactively-labeled decamethonium and carbamylcholine enter skeletal muscle fibers at the endplate region, apparently by penetrating AChR channels (Creese and England, 1970). A recent brief communication has reported that Sub and ACh apparently block AChR channels at the frog neuromuscular junction (Adams and Colquhoun, 1983; see also Neher and Steinbach, 1978).

Agonists apparently block open channels through binding at a site distinct from the active site responsible for triggering activation and desensitization of the AChR channel. There is a 1,500-fold difference in the K_Ds for agonist block at zero membrane potential and for low affinity agonist binding or the initial rate of sodium influx (see Table I). Channel block is also distinct from desensitization since the K_D for desensitization is more than 4,000-fold lower than the K_D for block. Furthermore, we find that single-channel currents become grouped due to desensitization (Sakmann et al., 1980) at concentrations much lower than those that block channels. Finally, channel block is extremely short lived relative to desensitization.

In some respects our observations resemble those made by Miller (1982) in studies of a potassium channel from sarcoplasmic reticulum, reconstituted in planar lipid bilayer membranes. He found that monovalent quaternary ammonium compounds produced a fast block, whereas a slow block was produced by divalent compounds with long methylene chains joining the charged groups. In contrast to our observations on Sub-induced block, he found that the slow blocking compounds had twice the effective valence of a monovalent blocker, suggesting that both charged groups penetrate the membrane field. He also studied the relationship between effective valence and methylene chain length, and concluded that ~65% of the membrane field dropped across only ~8 Å of the channel length. If our data are

interpreted in the same way, 80% of the membrane field would drop across <18 Å of this ACh-receptor channel.

We have analyzed our data in terms of a sequential channel-blocking model. A possible alternative model is an allosteric one, in which a third agonist molecule binds to a site and results in the channel closing transiently (see Neher and Steinbach, 1978). We analyzed our data for ACh in terms of the simplest version of this alternative, Scheme II

$$A_E + R_o \underset{l(V)}{\overset{k(V)}{\rightleftharpoons}} R_o A \underset{n(V)}{\overset{m(V)}{\rightleftharpoons}} R_b A.$$

Scheme II

In Scheme II an agonist molecule binds to a site on a receptor with an open channel, R_o, to produce a conducting complex, R_oA. The channel then closes transiently to a nonconducting state, R_bA. Block results from separate binding (association constant $K[V] = k[V]/l[V]$ and isomerization steps (forward equilibrium constant $L[V] = m[V]/n[V]$). The ability of this model to describe the data for ACh was tested under two assumptions about the voltage dependence of the parameters. When both $K(V)$ and $L(V)$ were assumed to depend exponentially on membrane potential, the model could not describe the data well. However, the data could be described if K was assumed to be independent of potential and $L(V) = L_o \exp(C_1V + C_2V^2)$ (see Stevens, 1978). In this case, $K = 1\ M^{-1}$ (dissociation constant 1,000 mM), $L_o = 7.7$, $C_1 = -0.060\ mV^{-1}$, and $C_2 = 1.92 \times 10^{-4}\ mV^{-2}$. If the membrane field is assumed to drop across 50 Å, these values correspond to an effective dipole moment change for the forward isomerization reaction of about −180 D and a polarizability of ~170,000 Å^3 (a change in the dipole of 1.1 D per mV change in membrane polarization). For comparison, at *Rana temporaria* junctions the channel closing rate has a voltage sensitivity corresponding to an effective dipole moment change of 33 D and a polarizability of 43,000 Å^3(−0.3 D/mV) (Neher and Stevens, 1979). Our data do not allow us to determine whether channel blockade or an allosteric mechanism is the correct choice for the mechanism of action of agonists on currents through ACh receptor channels (see also Neher and Steinbach, 1978).

Our observations demonstrate an additional interaction between cholinergic agonists and the nicotinic AChR: channel block. These observations also define the concentration range over which single-channel kinetics may be analyzed simply in terms of receptor activation processes. For studies of receptor activation the concentration of Sub should be lower than 3 μM, which severely limits the usefulness of this agonist. The most suitable agonist we have examined is ACh, with a nonblocking concentration range between 10 nM and 300 μM at −70 mV. The nonblocking range for carbamylcholine is between 1 μM and 1 mM.

We have observed that ACh blocks significantly at concentrations as low as 300 μM at −100 mV. If the receptors we have studied are similar in this property to other AChRs, blocking concentrations of ACh would probably be reached during neuromuscular transmission (Steinbach and Stevens, 1976), although block is unlikely to be of physiological significance due to its strong voltage dependence. Agonist block of channels must be accounted for, however, in biophysical studies of receptor activation. It is difficult to disentangle the actions of agonist to activate, desensitize, and block receptors in measurements of macroscopic membrane currents. Channel block can affect both the time course and peak amplitudes of endplate currents in voltage-clamped muscle fibers. Similarly, block would produce a voltage-dependent apparent saturation in studies of dose-response relationships, which measure macroscopic currents. Similar effects can be expected in studies of agonist-elicited ion fluxes, and in biochemical studies of rapid ligand binding to the AChR. Finally, other agonists may be more effective at blocking currents through the channels that they open (as Sub is more effective than is ACh). This possibility must be borne in mind when interpreting macroscopic dose-response relationships in terms of molecular models of receptor activation.

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