Voltage Dependence of Acetylcholine Receptor Channel Gating in Rat Myoballs

LEE D. CHABALA

From the Department of Medicine, Division of Environmental Medicine and Toxicology, Jefferson Medical College, Philadelphia, Pennsylvania 19107

ABSTRACT Whole-cell currents from nicotinic acetylcholine receptor (AChR) channels were studied in rat myoballs using a light-activated agonist to determine the voltage dependence of the macroscopic opening and closing rate constants. Myoballs were bathed in a solution containing a low concentration of the inactive isomer of the photoisomerizable azobenzene derivative, cis-Bis-Q. A light flash was then presented to produce a known concentration jump of agonist, trans-Bis-Q, across a wide range of membrane potentials in symmetrical solutions (NaCl or CsCl on both sides) or asymmetrical solutions (NaCl in the bath and CsCl in the pipette). At the low agonist concentration used in this study, the reciprocal of the macroscopic time constants gives an unambiguous measure of the effective closing rate. It showed an exponential decrease with membrane hyperpolarization between +20 and -100 mV, but tended to level off at more depolarized and at more hyperpolarized membrane potentials. The relative effective opening rate was derived from the steady-state conductance, the single-channel conductance, and the apparent closing rate; it decreased sharply in the depolarizing region and tended to level off and then turn up in the hyperpolarizing region. The two effective rate constants were shown to depend on the first, second, and third power of membrane potential.

INTRODUCTION

A key issue in the gating of acetylcholine receptor (AChR) channels is understanding how transition rates governing principal conformations depend on membrane potential (Stevens, 1978; Neher and Stevens, 1979). Although some workers (Magleby and Stevens, 1972; Dionne and Stevens, 1975; Horn and Brodwick, 1980) found that both the macroscopic closing and opening rate constants were voltage dependent, other groups suggested that most of the voltage dependence was due to the apparent closing rate (Adams, 1976; Neher and Sakmann, 1975; Sheridan and Lester, 1975, 1977). Early work also suggested that agonist binding was rapid, whereas channel closing was rate limiting (Magleby and Stevens, 1972; Andersen and Stevens, 1973). More recent work, however, now suggests that receptor isomerization may be comparable to or faster than agonist dissociation, thus indicating that neither agonist binding nor receptor isomerization is rate limiting (Land, Salpeter, and...
A consequence of the rapid-isomerization hypothesis is that intermediate closed states, which are kinetically indistinguishable in the rapid-binding model, may be of functional importance. In light of this interpretation of agonist action at the AChR channel, the meaning of the macroscopic closing rate constant and its voltage dependence would change.

To examine more closely the voltage dependence of AChR channel gating, whole-cell and single-channel AChR currents were studied in voltage-clamped rat myoballs or in excised myoball patches. Kinetic and steady-state properties were studied across a wide range of membrane potentials using improved flash-induced agonist concentration jump techniques (Chabala, Gurney, and Lester, 1985, 1986).

Preliminary results have previously appeared in abstract form (Chabala and Lester, 1984, 1985).

**MATERIALS AND METHODS**

**Experimental Preparation and Solutions**

Small spherical myoballs (~20 μm in diameter) were prepared from primary cultures of neonatal rat thigh muscle as previously described (Chabala et al., 1986). The composition of the ionic solutions is given in Table I.

| TABLE I Composition of Solutions (Millimolar) |
|-----------------------------------------------|
| NaCl  | GluCl | MgCl₂ | CaCl₂ | Glucose | HEPES | EGTA | TTX  |
|-------|-------|-------|-------|---------|-------|------|------|
| Asymmetrical sodium-cesium solutions         |
| External 160 | 1.5 | 1.5 | 5 | 5 | 10⁻⁴ |
| Internal 140 | 1.5 | 1.5 | 5 | 5 | 10   |
| Symmetrical sodium solutions                 |
| External 140 | 1.5 | 1.5 | 35 | 5 | 10⁻⁴ |
| Internal 140 | 1.5 | 1.5 | 5 | 5 | 10   |
| Symmetrical cesium solutions                 |
| External 140 | 1.5 | 1.5 | 35 | 5 | 10⁻⁴ |
| Internal 140 | 1.5 | 1.5 | 5 | 5 | 10   |

**Voltage-Clamp Techniques**

Standard techniques were used to record whole-cell or single-channel currents. For details, see Chabala et al. (1986). Chamber temperature was maintained at 15 ± 0.1°C by two Peltier-driven thermoelectric devices with feedback from a linear thermistor (Chabala, Sheridan, Hodge, Power, and Walsh, 1985).

**Photochemical Techniques**

Photochemistry of the azobenzene Bis-Q molecule is described in Lester and Nerbonne (1982), while purification of the cis isomer is outlined in Nerbonne, Sheridan, Chabala, and Lester (1983). Optical calibration of the light-flash potency is given in Chabala et al. (1986). Flashes were imaged onto myoballs through the microscope camera port and objective from a xenon
short-arc flashlamp, which was mounted in a shielded cage above the microscope. For details, see Chabala et al. (1986).

**Macroscopic Gating Scheme**

Voltage-clamped myoballs were bathed in a solution containing 500 nM *cis*-Bis-Q (the inert isomer). A 50-μm-diam light flash then produced a well-localized jump in the concentration of *trans*-Bis-Q, which is an ACh agonist. In this case, the agonist concentration jump was 0 → 270 nM *trans*-Bis-Q (Chabala et al., 1986). After the agonist concentration jump, *trans*-Bis-Q would diffuse away into the reservoir of the chamber. Many such concentration jumps could be carried out without increasing the background conductance or inducing receptor desensitization.

The major component of the agonist-induced current followed a single exponential relaxation; thus, the macroscopic gating behavior was described by two kinetically distinguishable states,

\[
C \xrightarrow{\alpha'} O
\]

where \(C\) is the closed state, \(O\) is the open state, \(\beta'\) is the effective opening rate, and \(\alpha'\) is the effective closing rate. The prime symbols indicate that the rate constants are, in fact, "effective" rate constants that encompass the microscopic rate constants of a more mechanistically realistic kinetic scheme. The time dependence of the current given that the channel was closed initially is given by

\[
I(t) = \frac{\beta'}{\alpha' + \beta'} \left[ 1 - \exp \left( -\frac{t}{\tau} \right) \right] (V - V_{rev})
\]

where \(I(t)\) is the current at time \(t\), \(\gamma\) is the open-channel conductance, \(N\) is the apparent number of available channels, \(P_o(\infty)\) is the steady-state probability of being in the open state, \(\tau\) is the macroscopic time constant, \(V\) is the membrane potential, and \(V_{rev}\) is the reversal potential. In terms of the rate constants of Scheme 1, \(P_o(\infty)\) and \(\tau\) are given by

\[
P_o(\infty) = \frac{\beta'}{\alpha' + \beta'}
\]

and

\[
\tau = \frac{1}{\alpha' + \beta'}
\]

In a series of dose–response studies, we found that the macroscopic time constants for 270 nM *trans*-Bis-Q were still in the low concentration range (Chabala et al., 1986); thus, \(\alpha' = 1/\tau\). The steady-state conductance, \(G(\infty)\), is given by

\[
G(\infty) = \gamma N P_o(\infty)
\]

If \(N\) or \(P_o(\infty)\) were known, the absolute value of \(\beta'\) could be derived from the measured quantities \(G(\infty)\), \(\gamma\), and \(\alpha'\) at each membrane potential. Although the value of \(N\) was not determined, it was assumed to be constant, while \(P_o(\infty)\) was assumed to reflect the voltage dependence of \(G(\infty)\). Using these assumptions, the voltage dependence of \(\beta'\), however, could still be derived by plotting the relative value of \(\beta'\) at each membrane potential. The approach that was used was to calculate \(N\) by assuming that \(P_o(\infty) = 0.01\) at \(-100\) mV from Eq. 4. The justification for \(P_o(\infty)\) was governed by single-channel experiments with 270 nM *trans*-Bis-Q where the probability of opening was found to be quite low, but the choice of \(P_o(\infty)\) does not affect the voltage dependence of \(\beta'\). Relative values of \(\beta'\) were then estimated (see Figs. 4, 6,
and 8) from the expression:

$$\beta' = \frac{G(\alpha)\alpha'}{\gamma N - G(\alpha)} \approx P_0(\alpha)\alpha'$$  \hspace{1cm} (5)

**Experimental Protocols**

In experiments with asymmetrical sodium-cesium solutions, flash-induced currents from -100 to +80 mV (see Fig. 2) were collected four to six times in each myoball. Experimental trials consisted of seven 100-ms voltage steps in 30-mV increments from a holding potential (HP) of 0 mV in which the flash lamp was discharged 20 ms into each voltage step. 15 s was allowed for recovery between pulses. Macroscopic currents were averaged and fitted to a single exponential function (see Fig. 1 B) to estimate $\tau$ and the steady-state current, $I(\infty)$.

**FIGURE 1.** Analysis of agonist-induced whole-cell and single-channel currents. (A) Yellow-filter control trace and six agonist-induced current traces at +80 mV (HP = 0 mV) in symmetrical sodium solutions. The flash produced an agonist concentration jump of 0 → 270 nM trans-Bis-Q. The first arrow shows the start of the voltage step, while the second arrow indicates the flash. (B) Averaged difference current computed from six current traces and control trace in A. A semilogarithmic plot of fitted region and a superimposed single exponential fit ($\tau = 5.6$ ms) are shown above the trace. (C) Single-channel currents during 16 voltage steps to -100 mV (HP = 0 mV) in asymmetrical sodium-cesium solutions. Agonist concentration jump was 0 → 10.8 μM trans-Bis-Q. The first trace (inverted trace) is a yellow-filter control. The next 15 traces show single-channel currents. (D) Single-channel difference trace generated by subtraction of the first and second traces in episode. $T = 15^\circ$C.
The protocol for the symmetrical solutions involved recording currents from +110 to -160 mV in 30-mV increments. A yellow glass filter (e.g., Schott GG 435) was placed in the light path to block visible light on the first trial so that no agonist was produced. This yellow-filter control trial was followed by six or eight experimental trials (Fig. 1 A). 20–30 s recovery time was allowed between trials. Macroscopic "difference" currents were created by averaging experimental traces and subtracting the control trace (Fig. 1 B). The time constant for current rundown was determined from periodic retests at +80 mV, and \( I(\infty) \) values were corrected for the measured rundown.

Single-channel currents were evoked in outside-out patches using similar protocols in which a yellow-filter control trial preceded 15 experiments trails (Fig. 1 C). Single-channel difference currents (Fig. 1 D) were analyzed for amplitude information to construct the open-channel \( I-V \) relations. Those myoballs were bathed in a solution containing 20 \( \mu \)M \( \text{cis}-\text{Bis-Q} \), and the agonist concentration jump was 0 \( \rightarrow \) 10.8 \( \mu \)M \( \text{trans}-\text{Bis-Q} \).

**RESULTS**

**Experiments with Asymmetrical Ionic Solutions**

A family of flash-induced AChR channel currents in asymmetrical sodium-cesium solutions is shown in Fig. 2 A. Each agonist-induced current is the average of six relaxations at each membrane potential. The nonlinear \( I(\infty)-V \) relation for seven myoballs is shown in Fig. 2 B, and the corresponding \( G(\infty)-V \) relation is illustrated in Fig. 2 C. Although the \( I(\infty)-V \) relation in Fig. 2 B looks fairly regular, it is clear from the \( G(\infty)-V \) curve that the conductance change through the reversal potential region is irregular.

Single-channel experiments in outside-out patches were carried out to examine more closely the origin of the bump through the reversal potential region of the \( G(\infty)-V \) curve. Typical flash-induced single-channel AChR currents are shown in Fig. 3 A from an excised myoball patch exposed to the same asymmetrical ionic conditions. Single-channel difference currents (as in Fig. 1 D) were used to determine the open-channel \( i-V \) relation shown in Fig. 3 B. The limiting slope conductance was \( \sim 31 \) pS at hyperpolarized potentials, while it was \( \sim 55 \) pS at depolarized potentials.

The open-channel conductance was normalized from -100 to +80 mV, and those values were used to normalize the \( G(\infty)-V \) curve (from Fig. 2 C). This largely eliminated the bump in the \( G(\infty)-V \) curve, and the normalized \( G(\infty)-V \) curve was found to show a smooth decrease with increasing depolarization throughout the voltage range (Fig. 4 A). The theoretical fit that describes the voltage dependence of the normalized \( G(\infty)-V \) curve in terms of the voltage dependence of \( \alpha' \) and \( \beta' \) is discussed below.

Each averaged macroscopic relaxation was fitted to a single exponential function (as shown in Fig. 1 B) to derive an estimate of the effective closing rate, \( \alpha' \). The estimates of \( \alpha' \) from seven myoballs were averaged and plotted against membrane potential as shown in Fig. 4 B. Although the logarithm of \( \alpha' \) depended linearly on membrane potential from +20 to -100 mV, it tended to level off at more positive potentials. The voltage dependence of \( \alpha' \), however, is not sufficient to account for the voltage dependence of \( G(\infty) \), which suggests that the effective opening rate, \( \beta' \), is also voltage dependent. Relative values of \( \beta' \) were derived from measured values of \( G(\infty) \), \( \gamma \), and \( \alpha' \) as discussed in Materials and Methods. As illustrated in Fig. 4 B, the
FIGURE 2. $I(\infty)-V$ and $G(\infty)-V$ relations in asymmetrical sodium-cesium solutions. (A) Seven agonist-induced currents from $-100$ to $+80$ mV (HP = 0 mV) with agonist conditions as described in Fig. 1 A. Each current trace is the average of six relaxations in the same myoball. (B) Average $I(\infty)-V$ relation from seven myoballs. Each data point is the mean (±SEM) of seven averaged current traces, each of which was generated by averaging four to six relaxations. The smooth curve is a cubic spline fit used to estimate $V_{\text{rev}}$ ($\sim +7$ mV). (C) Average $G(\infty)-V$ relation for data from B. $T = 15^\circ C$.

FIGURE 3. Single-channel currents in asymmetrical sodium-cesium solutions. (A) 15 superimposed traces are shown at $+140$ mV (upper traces) and at $-130$ mV (lower traces), with HP = 0 mV and agonist conditions as in Fig. 1 C. (B) Open-channel $i-V$ relationship from $-190$ to $+140$ mV. Cubic spline fit was used to estimate $V_{\text{rev}}$ ($\sim +5$ mV). Slope conductances for hyperpolarizing and depolarizing regions are $\sim 31$ and $\sim 55$ pS, respectively. $T = 15^\circ C$. 
logarithm of $\beta'$ showed a more or less linear dependence on membrane potential in the depolarizing region but tended to level off at more hyperpolarized values. The voltage dependence of $\beta'$ was basically the inverse of that found for $\alpha'$. The smooth curves that describe the voltage dependence of $\alpha'$ and $\beta'$ are discussed below.

![Graph A]

FIGURE 4. Normalized $G(\infty)$-$V$ relation and voltage dependence of $\alpha'$ and $\beta'$ in asymmetrical ionic solutions. (A) Normalized $G(\infty)$-$V$ relation after dividing the data of Fig. 2B by the normalized conductance from Fig. 3. Theoretical fit is given by Eq. 4 with $\gamma = 31$ pS and $N = 22,000$. (B) Voltage dependence of $\alpha'$ (filled circles) and $\beta'$ (filled squares). Theoretical fits are given by Eq. 8.

Experiments with Symmetrical Ionic Solutions

A partial family of averaged whole-cell difference currents obtained in symmetrical sodium conditions is shown in Fig. 5A. On this scale (as in Fig. 1A), it is clear that
outward agonist-induced currents, which appear tiny in comparison to the larger inward currents (as in Fig. 2A), are in fact well resolved. The complete \( I(\infty)-V \) relation for 19 myoballs in symmetrical sodium solutions is shown in Fig. 5B. Flash-induced single-channel currents (under the same ionic conditions) are shown in Fig. 5C, while the complete \( i-V \) relation, which was determined from difference currents (as in Fig. 1D), is shown in Fig. 5D. Except for the slight rectification at large positive potentials, the \( i-V \) relation was linear with a slope conductance of \(~29\) pS.

The \( G(\infty)-V \) relation for the symmetrical sodium data is shown in Fig. 6A. Since the \( i-V \) relation was essentially linear, it was not necessary to normalize the \( G(\infty)-V \) data. The voltage dependence of both \( \alpha' \) and \( \beta' \) is shown in Fig. 6B. As in the case of asymmetrical ionic conditions, the logarithm of \( \alpha' \) showed a linear dependence on

---

**Figure 5.** Agonist-induced currents in symmetrical sodium solutions. (A) Difference currents (HP = 0 mV) with agonist conditions as in Fig. 1A. Each difference current is the average of eight current traces. (B) Complete average \( I(\infty)-V \) relation, where each data point represents the mean \((\pm SEM)\) of 19 averaged difference currents, each of which was based on six or eight relaxations. The smooth curve is a cubic spline fit. (C) Single-channel currents with agonist conditions as in Fig. 1C. 15 superimposed traces are shown at both +110 mV (upper traces) and at −100 mV (lower traces), with HP = 0 mV. (D) Single-channel difference traces (as in Fig. 1D) were used to construct the complete \( i-V \) relation. The conductance was fairly linear \((\gamma \approx 29 \text{ pS})\). \( T = 15^\circ\text{C} \).
membrane potential over a portion of the hyperpolarizing region, while it tended to saturate at each potential extreme. The logarithm of $\beta'$, however, showed a linear decrease in the depolarizing region but tended to level off and turn up in the hyperpolarizing region. The theoretical fits are described below.

![Diagram A](image-url)

**Figure 6.** $G(\infty)-V$ relation and voltage dependence of $\alpha'$ and $\beta'$ in symmetrical sodium solutions. (A) Average $G(\infty)-V$ relation from data in Fig. 5. (B) Voltage dependence of $\alpha'$ (filled circles) and $\beta'$ (filled squares). Error bars are smaller than symbols in some cases. Theoretical fits for the macroscopic conductance (Eq. 4, with $\gamma = 29$ pS and $N = 60,000$) and effective rate constants (Eq. 8) are shown.

Whole-cell experiments were also conducted in symmetrical cesium solutions, as shown in Fig. 7 A. The complete $I(\infty)-V$ relation from nine such myoballs is shown in Fig. 7 B. The currents were qualitatively similar to those recorded under symmetrical sodium solutions, although they were more than twofold smaller in comparable
myoballs that were cultured at the same time. Representative single-channel currents evoked in symmetrical cesium solutions are shown in Fig. 7 C, while the open-channel $i-V$ relation is shown in Fig. 7 D. The slope conductance was $\sim 42$ pS. The $G(\infty)-V$ curve for the symmetrical cesium data is shown in Fig. 8 A, while the corresponding plots of $\alpha'$ and $\beta'$ are shown in Fig. 8 B.

\[ a_{ij} = v \exp \left( -U_{ij}/kT \right) \]  

**Voltage Dependence of the Macroscopic Rate Constants**

According to the theory of absolute reaction rates (Eyring, 1935), transition rates for chemical reactions have the general form
where $a_{ij}$ is the rate constant for the transition from states $i$ to $j$, $v$ is a frequency factor (units of time$^{-1}$), $U_{ij}$ is the activation energy or barrier height for the transition state between states $i$ and $j$, $k$ is Boltzmann’s constant, and $T$ is the absolute temperature. The influence of membrane potential on such rate constants is mediated through the free energy term, $U_{ij}$, which in this case is determined by the permanent and induced effects of polar groups associated with the AChR molecule. The manner in which an electric field can influence a polarizable molecule has been explored in detail by Debye (1929). A similar approach has also been applied to the study of gating in ion
TABLE II

Coefficients of Effective Rate Constants

|       | $\alpha'$ | $\beta'$ | $A$ (ms$^{-1}$) | $B$ (mV$^{-1}$) | $C$ (mV$^{-2}$) | $D$ (mV$^{-3}$) |
|-------|-----------|----------|----------------|----------------|----------------|----------------|
| Asymmetrical sodium-cesium experiments ($n = 7$) | 1.63 $\times$ $10^{-1}$ | 7.61 $\times$ $10^{-5}$ | $-1.92 \times 10^{-5}$ | $-1.62 \times 10^{-7}$ |
| $\alpha'$ | 4.73 $\times$ $10^{-4}$ | $-6.48 \times 10^{-5}$ | $-8.38 \times 10^{-6}$ | $1.11 \times 10^{-7}$ |
| Symmetrical sodium experiments ($n = 19$) | 1.58 $\times$ $10^{-1}$ | 5.98 $\times$ $10^{-5}$ | $-1.41 \times 10^{-5}$ | $-7.51 \times 10^{-8}$ |
| $\beta'$ | 6.84 $\times$ $10^{-4}$ | $-4.17 \times 10^{-5}$ | $-3.59 \times 10^{-5}$ | $-1.23 \times 10^{-7}$ |
| Symmetrical cesium experiments ($n = 9$) | 1.72 $\times$ $10^{-1}$ | 6.59 $\times$ $10^{-5}$ | $-6.21 \times 10^{-6}$ | $-8.50 \times 10^{-8}$ |
| $\alpha'$ | 8.51 $\times$ $10^{-4}$ | $-2.75 \times 10^{-5}$ | $-2.91 \times 10^{-5}$ | $-1.12 \times 10^{-7}$ |

Each effective rate constant has the following form: $\alpha', \beta' = A \exp (BV + CV^2 + DV^3)$ where $\alpha'$ is the effective closing rate, $\beta'$ is the effective opening rate, and the coefficients, which are described in the text, have the indicated values.

TABLE III

Apparent Polarizability Coefficients

|       | $\alpha'$ | $\beta'$ | $\mu$ (debye) | $b$ (nm$^2$) | $c \times 10^{-34}$ e.s.u. |
|-------|-----------|----------|---------------|--------------|--------------------------|
| Asymmetrical sodium-cesium experiments | 36.1 | 4.19 | 7.43 |
| $\alpha'$ | 31.0 | 5.18 | 5.99 |
| Symmetrical sodium experiments | 28.6 | 3.78 | 3.44 |
| $\alpha'$ | 19.9 | 5.16 | 5.64 |
| Symmetrical cesium experiments | 31.5 | 2.87 | 3.90 |
| $\alpha'$ | 13.1 | 4.81 | 5.14 |

The apparent polarizability coefficients ($\mu$ for orientation polarization, $b$ for molecular polarization, and $c$ for hyperpolarization) were derived as described in the text from the corresponding coefficients in powers of $V$ (i.e., $A$, $B$, $C$, and $D$) from the effective closing and opening rate constants in Table II. Note, 1 debye = $10^{-18}$ e.s.u.
rate constants is given by

$$a_{ij}(V) = \exp\left(-\frac{U_{ij}(0) - \frac{V^2}{2m} \mu_j - \frac{V^3}{6m} \epsilon_j}{kT}\right)$$  \hspace{1cm} (7)$$

where \(m\) is the effective membrane thickness, \(U_{ij}(0)\) is the free energy associated with the transition conformation between states \(i\) and \(j\) at \(V = 0\), \(\mu_j\) is the effective dipole moment, \(\epsilon_j\) is the molecular polarization coefficient, and \(\epsilon_j\) is the hyperpolarization coefficient. For additional details, see Stevens (1978). In terms of fitting the voltage dependence, however, a more appropriate expression is given by

$$a_{ij}(V) = A \exp(BV + CV^2 + DV^3)$$  \hspace{1cm} (8)$$

where \(A\) is the value of the rate constant at \(V = 0\), and so forth.

The coefficients \(A, B, C,\) and \(D\) were determined from a Marquardt least-squares fit (cf. Bevington, 1969) of the voltage dependence for both \(\alpha'\) and \(\beta'\). The fitted values are given in Table II. Theoretical fits of the voltage dependence of \(\alpha'\) and \(\beta'\) from Eq. 8 are shown in Figs. 4, 6, and 8. The voltage dependence of each effective rate constant was fitted fairly well except for the tendency for \(\alpha'\) to miss the rolloff in the extreme hyperpolarizing region in symmetrical sodium solutions (Fig. 6 B).

The theoretical fits of \(\alpha'\) and \(\beta'\), along with measured values of \(\gamma\) and estimated values for \(N\), were also used to model the voltage dependence of \(G(\infty)\) using Eqs. 2 and 4 (see Materials and Methods). As shown in Figs. 4, 6, and 8, the voltage dependence of \(\alpha'\) and \(\beta'\) account fairly well for the nonlinear \(G(\infty)-V\) curves, except for the slight tendency for the conductance to roll off at extreme hyperpolarized potentials in symmetrical salt solutions. That rolloff and the rolloff in \(\alpha'\), however, could be accounted for by including a term that depends on \(V^4\) in \(\alpha'\), but it may also be due in part to uncompensated series resistance, and there was less of a rolloff in symmetrical cesium solutions (Fig. 8 A) where the macroscopic currents were smaller. The time course of the macroscopic current was also well accounted for at all membrane potentials (not shown).

The apparent polarizability coefficients (\(\mu, b,\) and \(c\)) were calculated for \(\alpha'\) and \(\beta'\) by equating the coefficients of corresponding powers of \(V\) from Eqs. 7 and 8, as in the case of \(\mu = mBkT\), etc. An effective average membrane thickness of 4.0 nm was used in the calculations, which are summarized in Table III. Although the magnitude of \(\beta'\) (i.e., the preexponential coefficient \(A\)) was determined only to a multiplicative constant, the apparent polarizability coefficients, which reflect the voltage dependence, should be accurate. The units of \(\mu\), the permanent dipole moment term, are given in debye. The constant \(b\), the molecular polarizability term, has units of volume and corresponds roughly to the apparent volume of the polarized macromolecule. The hyperpolarizability constant, \(c\), is given in electrostatic units (e.s.u.). These constants reflect linear and inductive effects of polar groups associated with the AChR channel. Although both \(\alpha'\) and \(\beta'\) showed a nonexponential voltage dependence, it is not clear whether this reflects the intrinsic voltage dependence of the microscopic closing and opening rate constants or whether it is due to an interaction of several microscopic rate constants (cf. below).
DISCUSSION

Voltage Dependence of the Effective Transition Rates

The principal conclusion from this work is that the effective transition rates, $\alpha'$ and $\beta'$, which control voltage-sensitive gating in the rat myoball nicotinic AChR channel, exhibit higher-order voltage dependence. $\alpha'$ decreases exponentially over part of the hyperpolarizing region but tends to level off or saturate at more depolarized and at more hyperpolarized potentials. In contrast, $\beta'$ decreases exponentially in the depolarizing region and tends to level off and perhaps turn up in the hyperpolarizing region. The effective transition rates were shown to depend on the first, second, and third powers of membrane potential as though the charged or polar groups associated with the effective channel-gating mechanism were subject to orientation polarization, molecular polarization, and hyperpolarization (cf. Buckingham and Pople, 1955; Le Fèvre, 1965).

If the voltage dependence of $\alpha'$ arises from a polarization of charged or polar groups during the conformational change as open channels close, one would expect a counter charge movement to influence $\beta'$ as closed channels open (Magleby and Stevens, 1972; Andersen and Stevens, 1973; Dionne and Stevens, 1975; Stevens, 1978; Neher and Stevens, 1979). Since the voltage dependence of $\beta'$ was approximately the inverse of that observed for $\alpha'$, the macroscopic receptor gating mechanism appears to be modulated by a roughly symmetrical energy barrier between the functional open and closed conformations.

Relationship between Macroscopic and Microscopic Rate Constants

To look more closely at the significance of the voltage dependence of the macroscopic transition rates, it is instructive to consider a more accurate model of sequential receptor activation. A plausible model, which accounts for most of the macroscopic conductance (Adams, 1975; Dionne, Steinbach, and Stevens, 1978; Dreyer, Peper, and Sterz, 1978; Sheridan and Lester, 1977; Chabala et al., 1986; Sine and Steinbach, 1986), is the following four-state scheme:

\[
2A + T \xrightarrow{k_1} A + AT \xrightarrow{k_2} A + AT \xrightarrow{\alpha} A_R
\]

where $T$ and $R$ represent closed and open conformations, respectively, and $A$ represents the agonist. The association rate constants are $k_1$ and $k_2$, the dissociation rate constants are $k_{-1}$ and $k_{-2}$, the opening rate constant is $\beta$, and the closing rate constant is $\alpha$.

The macroscopic rate constants of Scheme 1 are related to the microscopic rate constants of Scheme 2 as follows:

\[
\beta' = \frac{2k_1k_2A^2\beta}{2k_1k_2A^2 + 4k_1k_{-2}A + 2k_{-1}k_{-2}}
\]  

and

\[
\alpha' = \frac{2k_{-1}k_{-2}A}{\alpha k_{-1} + \alpha k_{-2}A + \beta k_{-1} + \beta k_{-2} + 2k_{-1}k_{-2}}
\]
where $\beta'$ is the true opening rate ($\beta$) multiplied by the fraction of occupied closed channels that can open (cf. Colquhoun, 1981), and $\alpha'$ is the reciprocal mean waiting time (cf. Feller, 1968) for a transition that starts in state $A_2R$ and exits to state $T$. The latter waiting time would dominate the slow component of a macroscopic relaxation (Colquhoun and Hawkes, 1981). The measured burst length is somewhat shorter than the calculated burst length, however, because of the time spent in the nonconductive $A_2T$ and AT states before entering the T state to terminate the burst (Colquhoun and Hawkes, 1981), but that detail will not be considered here because the correction is very small.

The low-concentration time constant (i.e., $\tau = 1/\alpha'$), or $\tau = 1/\alpha$ as above, which was previously referred to as the lifetime of the open state (Neher and Sakmann, 1975, 1976; Sheridan and Lester, 1975; Sakmann and Adams, 1978; Neher and Stevens, 1979), is now often referred to as the mean burst duration to reflect postulated multiple reopenings during a single agonist-receptor occupancy (Colquhoun and Sakmann, 1981, 1985). Confirmation of that hypothesis, however, is still lacking (Colquhoun, Ogden, and Cachelin, 1986), and there is a different interpretation of the brief gaps (Sine and Steinbach, 1986), so it is not yet clear if the closed-occupied states are functionally important.

In the case of rapid binding and slow isomerization, the interpretation of the voltage dependence of $\alpha'$ and $\beta'$ would be straightforward (i.e., $\alpha' = \alpha$ and $\beta' = \beta$), although it is clear that the association rates would have to approach values for a diffusion-limited reaction between asymmetric molecular species (cf. Shoup, Lipari, and Szabo, 1981). Colquhoun and Sakmann (1981, 1985), however, proposed that the brief gaps observed during channel opening may reflect steps in receptor activation. According to that hypothesis, the isomerization step would no longer be rate limiting, and in that case the voltage dependence of $\alpha'$ and $\beta'$ would be more difficult to interpret. Assuming that the brief gaps are not due to undetected channel block by the agonist (as in the case of carbachol; Colquhoun et al., 1986) and that $\beta$ is in fact fairly large (but see Sine and Steinbach, 1986), the denominators of Eqs. 9 and 10 would be dominated by the terms $k_{-1}k_{-2}$ and $\beta k_{-1}$, respectively, so

$$\beta' = \frac{k_1k_2A^2\beta}{k_{-1}k_{-2}} \quad (11)$$

and

$$\alpha' = \frac{2k_{-2}\alpha}{\beta} \quad (12)$$

Although this is clearly an empirical matter, one could imagine several ways in which the ratio $k_1k_2/k_{-1}k_{-2}$ in Eq. 11 could remain voltage independent such that the voltage dependence of $\beta'$ could in fact reflect the voltage dependence of $\beta$. With regard to Eq. 12, Leibowitz and Dionne (1984) showed that $k_{-2}$ and $\beta$ have similar magnitudes (in garter snake) that increase with hyperpolarization. The ratio $k_{-2}/\beta$ would then be roughly constant, and the voltage dependence of $\alpha'$ would be due largely to the voltage dependence of $\alpha$.

Although several groups have used single-channel techniques to estimate two or
three of the microscopic rate constants (i.e., $\alpha$, $\beta$, and $k_{-2}$), there has yet to develop any sort of consensus agreement with regard to the voltage dependence of either $\beta$ or $k_{-2}$ (cf. Leibowitz and Dionne, 1984; Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986; Colquhoun and Ogden, 1988; Sine, Claudio, and Sigworth, 1990), and it is unclear if any of those rate constants show saturation effects. There is also disagreement regarding the magnitude of $\beta$, and the issue of whether or not $\beta$ depends on the agonist is still unresolved (Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986). Some of the disagreements may be due to receptor subunit composition (Auerbach and Lingle, 1987; Steinbach, 1989; Sine et al., 1990), but this is not yet clear. Finally, there is very little information regarding the voltage dependence of $k_1$, $k_2$, or $k_{-1}$ (but see Sine et al., 1990), although as suggested by Eq. 11, one or more of those rate constants must be voltage dependent if $\beta$ and $k_{-2}$ do have a similar voltage dependence. Once the voltage dependence of the microscopic transition rates is better understood, however, one would expect to be able to predict the voltage dependence of the macroscopic transition rates from Eqs. 9–12.

Comparison with Previous Work

The effective closing rates of frog ($Rana pipiens$) or toad endplate currents have been shown to decrease with membrane hyperpolarization (Gage and Armstrong, 1968; Kordaš, 1969; Gage and McBurney, 1972; Magleby and Stevens, 1972; Andersen and Stevens, 1973). When the experiments were repeated in the frog $Rana temporaria$, however, the estimates of $\alpha'$ were shown to decrease exponentially in the hyperpolarization region and to saturate in the depolarizing region (Stevens, 1978; Neher and Stevens, 1979). Marchais and Marty (1979) also found a nonexponential relation between the time constants of AChR channel currents (in Aplysia neurones) and membrane potential; those time constants leveled off in the hyperpolarizing region. The voltage dependence of $\alpha'$ has also been studied in the AChR channel in rat myoballs by Horn and Brodwick (1980). They found that $\alpha'$ decreased exponentially at hyperpolarized potentials but showed a tendency to level off at $+40 \text{ mV}$. In this article, which explored a wider potential range, it was shown that $\alpha'$ in rat myoballs does indeed decrease exponentially over a portion of the hyperpolarizing range, but tends to saturate at both potential extremes.

By way of contrast, it has been found by some workers that the macroscopic opening rate has little if any voltage dependence (Neher and Sakmann, 1975; Sheridan and Lester, 1975, 1977; Adams, 1976), although most estimates were made in the hyperpolarizing region, where $\beta'$ may tend to level off. Magleby and Stevens (1972) and Dionne and Stevens (1975), however, found that the opening rate shows a modest exponential decrease with membrane hyperpolarization. In the equation given by Dionne and Stevens (1975) for endplate conductance the opening rate is divided by the dissociation constant, so it was not possible to distinguish unambiguously between the effect of potential on agonist binding versus channel opening. Although those authors chose to assign the residual voltage dependence of the peak endplate currents to the opening rate, it may represent the voltage dependence of agonist binding. Finally, Horn and Brodwick (1980) estimated the voltage dependence of $\beta'$ in rat myoballs from the difference in slopes for conductance and $\alpha'$ and obtained a slope for $\beta'$ that agreed with the value reported by Dionne and Stevens.
Effects of Cesium Ions

Macroscopic currents in symmetrical cesium solutions were about twofold smaller compared with those in symmetrical sodium solutions although the conductance was 1.4-fold larger. That effect could arise if $\beta'$ and thus $P_\alpha(\infty)$ were about threefold smaller in symmetrical cesium experiments. A reduction in $\beta'$ could occur through an effect of cesium ions on one of the microscopic rate constants, in Eq. 9, or external cesium ions could act as a competitive antagonist as previously suggested (Hirano, Kidokoro, and Ohmori, 1987; Mathie, Cull-Candy, and Colquhoun, 1991).

There was, however, a clear effect of cesium ions on the apparent closing rate. The estimates of $\alpha'$ in symmetrical cesium solutions were generally larger than in symmetrical sodium or asymmetrical sodium-cesium solutions, so the effect appears to be due to external cesium ions. A possible mechanism is that external permeant cations influence the open conformation by binding to a site in the channel that modulates channel closing (Ascher, Marty, and Heild, 1978; Gage and Van Helden, 1979; Marchais and Marty, 1979).

Cesium ions also influenced permeation. The conductance in symmetrical cesium solutions ($\gamma = 42 \ pS$) was ~1.4-fold larger than it was in symmetrical sodium solutions ($\gamma = 29 \ pS$). A similar symmetrical cesium conductance ratio was reported by Dani and Eisenman (1987) in rat myotubes. The cesium conductance in the asymmetrical sodium-cesium experiments, however, was ~55 pS. A comparable large cesium conductance (at a lower cesium concentration) in sodium-cesium experiments has been reported by Lo, Pinkham, and Stevens (1990) for BC3H-1 cells. Although the observations on cesium conductance were not studied in detail, they might be expected in a two-site barrier model with multiple ion occupancy.

The author would like to thank Dr. Henry A. Lester, in whose laboratory this work was carried out. This project could not have been carried out without his pioneering work with photochemical techniques, and the author would like to acknowledge his participation in early phases of the work and for his continued support and encouragement. The author would also like to acknowledge helpful discussions with Dr. Manuel Covarrubias and with Dr. Richard Horn.

The work was supported in part by NIH grant NS-11756 (to H. A. Lester).

Original version received 22 January 1992 and accepted version received 20 July 1992.

REFERENCES

Adams, P. R. 1975. An analysis of the dose-response curve at voltage-clamped frog end-plates. \textit{Pflügers Archiv}. 360:145–153.

Adams, P. R. 1976. Voltage dependence of agonist responses at voltage-clamped frog end-plates. \textit{Pflügers Archiv}. 361:145–151.
Andersen, C. R., and C. F. Stevens. 1973. Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *Journal of Physiology*. 235:655–691.

Ascher, P., A. Marty, and T. O. Neild. 1978. Life time and elementary conductance of the channels mediating the excitatory effects of acetylcholine in *Aplysia* neurones. *Journal of Physiology*. 278:177–206.

Auerbach, A., and C. J. Lingle. 1987. Activation of the primary modes of large- and small-conductance cholinergic ion channels in *Xenopus* myocytes. *Journal of Physiology*. 395:437–466.

Buckingham, A. D., and J. A. Pople. 1955. Theoretical studies of the Kerr effect. I. Deviations from a linear polarization law. *Proceedings of the Physical Society*. 68A:905–909.

Chabala, L. D., A. M. Gurney, and H. A. Lester. 1985. Photoactivation and dissociation of agonist molecules at the nicotinic acetylcholine receptor channel in voltage-clamped rat myoballs. *Biophysical Journal*. 48:241–246.

Chabala, L. D., A. M. Gurney, and H. A. Lester. 1986. Dose-response of acetylcholine receptor channels opened by a flash-activated agonist in voltage-clamped rat myoballs. *Journal of Physiology*. 371:407–453.

Chabala, L. D., and H. A. Lester. 1984. Voltage dependence of acetylcholine receptor channel opening in rat myoballs. *Journal of General Physiology*. 84:24a–25a. (Abstr.)

Chabala, L. D., and H. A. Lester. 1985. Kinetic and steady-state properties of acetylcholine receptor channels in voltage-clamped rat myoballs. *Biophysical Journal*. 47:257a. (Abstr.)

Chabala, L. D., R. E. Sheridan, D. C. Hodge, J. N. Power, and M. P. Walsh. 1985. A microscope stage temperature controller for the study of whole-cell or single-channel currents. *Pflügers Archiv*. 404:374–377.

Colquhoun, D. 1981. How fast do drugs work? In *Towards Understanding Receptors*. J. W. Lamble, editor. Elsevier, Amsterdam. 16–27.

Colquhoun, D., and A. G. Hawkes. 1981. On the stochastic properties of single ion channels. *Proceedings of the Royal Society of London, Series B*. 211:205–235.

Colquhoun, D., and D. C. Ogden. 1988. Activation of ion channels in the frog end-plate by high concentrations of acetylcholine. *Journal of Physiology*. 395:131–159.

Colquhoun, D., D. C. Ogden, and A. B. Cachelin. 1986. Mode of action of agonists on nicotinic receptors. In *Ion Channels in Neural Membranes*. J. M. Ritchie, R. D. Keynes, and L. Bolls, editors. Alan R. Liss, Inc., New York. 255–273.

Colquhoun, D., and B. Sakmann. 1981. Fluctuations in the microsecond time range of the current through single acetylcholine receptor ion channels. *Nature*. 246:464–466.

Colquhoun, D., and B. Sakmann. 1985. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. *Journal of Physiology*. 369:501–557.

Dani, J. A., and G. Eisenman. 1987. Monovalent and divalent cation permeation in acetylcholine receptor channels: ion transport related to structure. *Journal of General Physiology*. 89:959–983.

Debye, P. 1929. Polar Molecules. Dover Publications, New York. 15–35.

Dionne, V. E., J. H. Steinbach, and C. F. Stevens. 1978. An analysis of the dose-response relationship at voltage-clamped frog neuromuscular junctions. *Journal of Physiology*. 281:421–444.

Dionne, V. E., and C. F. Stevens. 1975. Voltage dependence of agonist effectiveness at the frog neuromuscular junction: resolution of a paradox. *Journal of Physiology*. 251:245–279.

Dreyer, F., K. Peper, and R. Sterz. 1978. Determination of dose-response relationship at voltage-clamped frog neuromuscular junctions. *Journal of Physiology*. 281:395–419.

Eyring, H. 1935. The activated complex in chemical reactions. *Journal of Chemical Physics*. 3:107–115.
Feller, W. 1968. An Introduction to Probability Theory and Its Application. Vol. 2. 2nd ed. John Wiley & Sons, Inc., New York. 17–20.

Gage, P. W., and C. M. Armstrong. 1968. Miniature end-plate currents in voltage-clamped muscle fibre. *Nature*. 218:363–365.

Gage, P. W., and R. N. McBurney. 1972. Miniature end-plate currents and potentials generated by quanta of acetylcholine in glycerol-treated toad sartorius fibres. *Journal of Physiology*. 226:79–94.

Gage, P. W., and D. Van Helden. 1979. Effects of permeant monovalent cations on end-plate channels. *Journal of Physiology*. 288:509–528.

Hirano, T., Y. Kidokoro, and H. Ohmori. 1987. Acetylcholine dose-response relation and the effect of cesium ions in the rat adrenal chromaffin cell under voltage clamp. *Pflügers Archiv*. 408:401–407.

Horn, R., and M. S. Brodwick. 1980. Acetylcholine-induced current in perfused rat myoballs. *Journal of General Physiology*. 75:297–321.

Kirkwood, J. G. 1939. The dielectric polarization of polar liquids. *Journal of Chemical Physics*. 7:911–919.

Kordal, M. 1969. The effect of membrane polarization on the time course of the end-plate current in frog sartorius muscle. *Journal of Physiology*. 204:493–502.

Land, B. R., E. E. Salpeter, and M. M. Salpeter. 1981. Kinetic parameters for acetylcholine interaction in intact neuromuscular junction. *Proceedings of the National Academy of Sciences, USA*. 78:7200–7204.

Le Fèvre, R. J. W. 1965. Molecular refractivity and polarizability. *Advances in Physical Organic Chemistry*. 3:1–90.

Leibowitz, M. D., and V. E. Dionne. 1984. Single-channel acetylcholine receptor kinetics. *Biophysical Journal*. 45:153–163.

Lester, H. A., and J. M. Nerbonne. 1982. Physiological and pharmacological manipulations with light flashes. *Annual Review of Biophysics and Bioengineering*. 11:151–175.

Lo, D. C., J. L. Finkham, and C. F. Stevens. 1990. Influence of the γ subunit and expression system on acetylcholine receptor gating. *Neuron*. 5:857–866.

Magleby, K. L., and C. F. Stevens. 1972. A quantitative description of end-plate currents. *Journal of Physiology*. 223:173–197.

Marchais, D., and A. Marty. 1979. Interaction of permeant ions with channels activated by acetylcholine in *Aplysia* neurones. *Journal of Physiology*. 297:9–45.

Mathie, A., S. G. Cull-Candy, and D. Colquhoun. 1991. Conductance and kinetic properties of single nicotinic acetylcholine receptor channels in rat sympathetic neurones. *Journal of Physiology*. 439:717–750.

Neher, E., and B. Sakmann. 1975. Voltage-dependence of drug-induced conductance in frog neuromuscular junction. *Proceedings of the National Academy of Sciences, USA*. 72:2140–2144.

Neher, E., and B. Sakmann. 1976. Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature*. 260:779–802.

Neher, E., and C. F. Stevens. 1979. Voltage-driven conformational changes in intrinsic membrane proteins. In The Neurosciences: Fourth Study Program. F. O. Schmitt and F. G. Worden, editors. MIT Press, Cambridge. 623–629.

Nerbonne, J. M., R. E. Sheridan, L. D. Chabala, and H. A. Lester. 1983. cis-3,3',-Bis-[α-(trimethylammonium)methyl] azobenzene (cis-Bis-Q): purification and properties at acetylcholine receptors of *Electrophorus* electroplaques. *Molecular Pharmacology*. 23:344–349.

Sakmann, B., and P. R. Adams. 1978. Biophysical aspects of agonist action at frog endplate. *Advances in Pharmacology and Therapeutics*. 1:81–90.

Sheridan, R. E., and H. A. Lester. 1975. Relaxation measurements on the acetylcholine receptor. *Proceedings of the National Academy of Sciences, USA*. 72:3496–3500.
Sheridan, R. E., and H. A. Lester. 1977. Rates and equilibria at the acetylcholine receptor of *Electrophorus* electroplaques. A study of neurally evoked postsynaptic currents and of voltage-jump relaxations. *Journal of General Physiology.* 70:187–219.

Shoup, D., G. Lipari, and A. Szabo. 1981. Diffusion-controlled bimolecular reaction rates. The effect of rotational diffusion and orientation constraints. *Biophysical Journal.* 36:697–714.

Sine, S. M., T. Claudio, and F. J. Sigworth. 1990. Activation of *Torpedo* acetylcholine receptors expressed in mouse fibroblasts. Single channel current kinetics reveal distinct agonist binding affinities. *Journal of General Physiology.* 96:395–437.

Sine, S. M., and J. H. Steinbach. 1986. Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by low concentrations of agonist. *Journal of Physiology.* 373:129–162.

Steinbach, J. H. 1989. Structural and functional diversity in vertebrate skeletal muscle nicotinic acetylcholine receptors. *Annual Review of Physiology.* 51:353–365.

Stevens, C. F. 1978. Interactions between intrinsic membrane protein and electric field. *Biophysical Journal.* 22:295–306.