Block of Neuronal Fast Chloride Channels by Internal Tetraethylammonium Ions

DOROTHEA Y. SANCHEZ and ANDREW L. BLATZ

From the Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235

ABSTRACT The classical potassium-selective ion channel blocker tetraethylammonium ion (TEA) was shown to block chloride-selective ion channels from excised surface membranes of acutely dissociated rat cortical neurons when applied to the formerly intracellular membrane surface. The patch voltage clamp method was used to record single channel currents from fast Cl channels in the presence of TEA. At the filtering cut-off frequencies used (3-12.4 kHz, -3 dB) the TEA-induced block appeared as a reduction in single channel current amplitude, which was interpreted as the result of extremely fast on and off rates for the blocking reaction. Under the conditions of these experiments, the magnitude of TEA block was independent of membrane potential. Analysis of dose–response experimental results suggests that TEA binding resulted in a partial block of these channels with an equilibrium dissociation constant of ~12–15 mM. Analysis of amplitude distributions in the absence and presence of TEA using the method of Yellen (1994. Journal of General Physiology. 84:157–186.) produced a similar equilibrium dissociation constant and provided a blocking rate constant of ~16,000 mM⁻¹s⁻¹ and an unblocking rate constant of ~200,000 s⁻¹. The distributions of open and closed interval durations were fit with a blocking scheme where TEA binds to the open kinetic state with the constraint that the channel must reenter the open state before TEA can dissociate. The increase in the mean lifetime of the open state could be well fit by this model, but the distribution of closed interval durations could not, suggesting a more complex underlying blocking mechanism.

INTRODUCTION

Quaternary ammonium compounds (QA’s), particularly tetraethylammonium ion (TEA) and its longer chain derivatives, have been used extensively as pharmacological blocking agents and to determine the physical structure of potassium-selective ion channels (for reviews see Stanfield, 1983; Hille, 1992). QA’s also interact with a
variety of ion channels that are not selective for K ions (Stanfield, 1983; Lukács and Moczydlowski, 1990; Wang, Simon, and Wang, 1991; Sanchez and Blatz, 1992).

The mechanism of block of K channels by QA's has been investigated at the single channel level in bilayer and patch-clamp studies. Depending on the tissue, channel, and application site, TEA and other QA's have been found to exhibit (a) extremely fast blocking kinetics that are unresolvable by existing techniques; (b) intermediate blocking kinetics that result in increased open channel current noise and barely resolved “flickery” open events; and (c) slower blocking kinetics where the mean lifetimes of the blocked states are long enough such that they can be accurately measured (e.g., Blatz and Magleby, 1984; Yellen, 1984; Villarroel, Alvarez, Oberhauser, and Latorre, 1988; Kirsch, Taglialetela, and Brown, 1991; Langton, Nelson, Huang, and Standen, 1991; Taglialetela, VanDongen, Drew, Joho, Brown, and Kirsch, 1991; Newland, Adelman, Tempel, and Almers, 1992; Sanchez and Blatz, 1992; Carl, Frey, Ward, Sanders, and Kenyon, 1993; Choi, Mossman, Aube, and Yellen, 1993). In cloned channels, it has been possible to exploit the differential block by TEA to locate the presumptive pore region in K channel primary structure (MacKinnon and Yellen, 1990; Yellen, Jurman, Abramson, and MacKinnon, 1991; Hartmann, Kirsch, Drew, Taglialetela, Joho, and Brown, 1991). Alterations in blocking kinetics with experimental structural alterations of the channel primary structure have also been used to determine regions of channels that are critical to their function (Choi et al., 1993).

In a previous study (Sanchez and Blatz, 1992) we demonstrated that the most widely used QA, TEA, blocks neuronal fast Cl channels with a dissociation constant of 11.8 mM when applied to the extracellular membrane surface. Although this blocking concentration may seem high, it is actually less than that of the TEA block of several K channels (Blatz and Magleby, 1984; Villarroel et al., 1988; Carl et al., 1993). In the present study, we demonstrate that TEA applied to the formerly intracellular membrane surface also blocks fast Cl channels in excised inside-out patches of acutely dissociated rat cortical neurons. Compared with block by TEAo, block by internal TEA is much less voltage dependent. Analysis of dose–response relationships suggests that the major binding reaction of TEA with the channel leads to a partially conducting blocked state. Amplitude and dwell time distributions were analyzed in an attempt to determine the underlying kinetic mechanism of block.

Parts of this study have appeared in preliminary form (Sanchez and Blatz, 1991).

MATERIALS AND METHODS

Most of the experimental methods for recording and analyzing currents from single fast Cl channels used in this study have been described elsewhere in detail (Blatz and Magleby, 1985, 1986a,b; Blatz, 1991; Sanchez and Blatz, 1992), and they will only be described here briefly.

Rat Cortical Neuron Isolation

Rat cortical neurons were isolated from slices of young (4–15-d-old) rat brains using the method of Kay and Wong (1986). Plugs of frontal and parietal cortical tissue ~500 μm thick and 1 mm in diameter were incubated in Pipes saline (solution compositions below) containing
trypsin (type II, 0.65 mg/ml; Sigma Immunochemicals, St. Louis, MO) for 0.5–1.0 h at 37°C). After trypsin digestion, the cortical plugs were washed four times at room temperature with Pipes saline. When needed, one to three plugs were dissociated into single-cell suspensions by trituration through a fire-polished Pasteur pipette. Neurons were identified by their characteristic morphologies, and they were used within 1 h of dissociation.

Single-Channel Recording

Currents through single fast Cl channels were recorded from excised, inside-out membrane patches using the patch clamp technique (Hamill, et al., 1981). Single-channel currents were recorded with Axopatch 1B and 200A amplifiers and stored on FM tape (Racal Store 4DS, 30 inches per second) or VCR tape (Instrutech VR10A). The current record was played at reduced tape speed into a microcomputer with a 14-bit analogue-to-digital converter (Instrutech VR10A) for off-line analysis. Except where noted, all currents were digitized at a sampling interval of 2.865 μs. The major analog filtering was performed at cutoff frequencies of 6.4 and 12.4 kHz (reported here as the −3 dB point) with an eight-pole Bessel filter (Ithaco). All experiments were carried out at room temperature (20–23°C).

Solutions

The composition (in mM) of the solutions used in these experiments were: Pipes saline: 120 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 25 glucose, 20 Pipes (piperazine-N,N'-bis-(2-ethanesulfonic acid), pH 7.0. Pipette solution was 140 KCl, 1 EGTA, 5 TES, pH 7.0. Internal solution was 1,000 KCl, 1 EGTA, 5 TES, pH 7.0. Tetraethylammonium was obtained from Sigma Immunochemicals (Cl) and Fluka (Br).

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Solutions bathing the formerly intracellular surface of the membrane patch were conveniently switched by placing the tip of the electrode containing the patch into a microchamber (Barrett, Magleby, and Pallotta, 1981) connected to an eight-way valve and eight solution reservoirs.

Measurements of Single-Channel Amplitudes and Amplitude Distributions

For amplitude measurements, the filtered, digitized raw current record was displayed on a video monitor and horizontal cursors were adjusted by eye to the fully closed and open channel current levels. Additionally, amplitude distributions were collected, and the closed and open current amplitudes were determined from these. Amplitude distributions were collected from the raw filtered, digitized current record by binning individual digitized points according to their amplitudes. Sweeps of 640 points that contained at least one open event were used to construct amplitude distributions. This procedure should have no effect on subsequent analysis, since amplitude distributions were not used to measure such kinetic parameters as open probability. Usually, every eighth sampled point was used for amplitude distributions that would be equivalent to an effective sampling interval of 21.18 μs. Parameters determined using shorter effective sampling intervals, up to the shortest of 2.648 μs, were essentially identical with those determined with the longer sampling intervals, and so to conserve computation time, the longer sampling intervals were routinely used for amplitude distributions.

Determining Blocking and Unblocking Rate Constants from Amplitude Distributions

The method described by Yellen (1984) was used to derive blocking and unblocking rate constants from all-points amplitude distributions. A β function of the form:

\[
f(y) = y^{(a-1)}(1-y)^{(b-1)}/B(a, b)
\]

(1)
where

$$B(a, b) = \int y^{a-1}(1 - y)^{b-1} \, dy,$$

$$a = k_b \tau,$$

and

$$b = k_b \tau$$

was fit to the experimentally observed amplitude distributions. $k_b$ and $k_b$ are the unblocking and blocking rate constants, respectively, in the simple two-state blocking reaction:

$$O \leftrightarrow B,$$

where $O$ represents the open kinetic state, and $B$ represents the blocked kinetic state. In this analysis, it is assumed that the single channel current level of the blocked state is 0, and that the current level of the open state is fully open.

For a given Bessel filter, $\tau$ can be determined empirically to be $0.228/f_c$, where $f_c$ is the $-3$ dB cut-off frequency of the low-pass filter. The normal sequence of analysis was to calculate an amplitude histogram from the raw current record, fit a normal distribution to the peak of current representing the closed channel state, and subtract the mean of this Gaussian distribution from the entire distribution. The current peak representing the current amplitude when a single CI channel is open in the presence of TEA was then normalized to the fully open amplitude previously determined for the unblocked channel. The resulting distribution, consisting of a peak between 0 and 1 was fit, using the Marquardt-Levenberg algorithm with Eq. 1 convolved with the amplitude distribution of the closed channel.

**Measurement of Durations of Open and Closed Intervals**

A 50% threshold method was used to determine the durations of open and closed intervals from the digitized current data. The current records were monitored visually during playback into the computer to prevent the inclusion of noise spikes and patch breakup events. Once digitized, the data were again examined, both visually and by using stability plots (see below), to ensure that baseline shifts, noise artifacts, and shifts in gating kinetics (moding) were not present in the portions of the experimental record used for analysis. Open and closed interval durations were measured as the elapsed time between 50% threshold crossings from the digitized data. At the 379 kHz sampling rate (1 point/2.648 ns) used in these experiments, the sampling errors described by McManus, Blatz, and Magleby (1987) are negligible.

"Stability plots" (Blatz and Magleby, 1986a; Weiss and Magleby, 1990; Blatz, 1991) were constructed and examined for all interval duration data sets by calculating the mean open or closed duration over subsets (usually containing 50–200 events) of the data record and plotting this as the running mean open or closed interval duration vs interval number.

**Fitting Kinetic Models to Single-Channel Data in the Presence of Blockers**

Methods described by Colquhoun and Sigworth (1983), Blatz and Magleby (1986a,b), McManus et al. (1987), McManus and Magleby (1991), and Weiss and Magleby (1990) were used to fit kinetic reaction schemes to the distributions of open, closed, and blocked single-channel interval durations in the absence and presence of TEA. The so-called Q-matrix approach of Colquhoun and Hawkes (1983) with the approximate corrections for missed events of Blatz and Magleby (1986a) was used in an iterative fashion to determine the minimum
number of kinetic states, rate constants, and transition pathway configurations required to adequately fit unconditional open and closed interval distributions.

RESULTS

TEA Reduces Single-Channel Current Amplitude

Current traces from a single CI channel in the presence of increasing concentrations of TEA are shown in Fig. 1. Selected records were collected from an excised inside-out membrane patch containing several fast CI channels. Unitary conductance under these conditions in the absence of TEA was ~140 pS. In the absence of blocker, fast CI channels exhibited typical single ion channel kinetic behavior (Blatz, 1991). Closed interval durations ranged from microseconds well into seconds to minutes, suggesting a multiplicity of closed kinetic states with mean lifetimes of microseconds to minutes. The range of open interval durations was much smaller, with most of the open events falling within a range of 0.1–5 ms with a mean open duration of ~0.3–0.6 ms at the filtering bandwidth of these experiments.

In the presence of millimolar concentrations of TEA, open channel currents were reduced in amplitude, such that the only transitions observed were between the fully closed state and an open state of reduced current. Transitions into the fully open

![Figure 1. Single neuronal fast CI channel currents recorded in the presence of increasing concentrations of TEA. TEA concentrations (in mM) are indicated above each current trace. Filtering cut-off frequency, 6.44 kHz; holding potential, —47 mV (K equilibrium potential). (dotted lines), Closed current level.](image-url)
state were never recorded. Two possible general mechanisms for such a current reduction are: (a) the TEA binding and unbinding rates could be very fast leading to a time-averaged reduction in single-channel current; or (b) TEA binding kinetics could be very slow such that the mean lifetime of the TEA-blocked state (which, in this case, would have to be an open-but-blocked state of reduced single-channel conductance) was much longer than the average length of an experiment, so that no unblocking events would be detected. Mechanism 2 is not very likely, based on the observation (not shown) that during the wash-in of solutions containing TEA, the amplitudes of the single-channel currents were reduced in a smooth manner rather than in the step-wise manner predicted by this mechanism. It is likely, then, that TEA blocking kinetics are very rapid, and the reductions in single channel current were

![Graph](image_url)

**Figure 2.** Dose-response relationship for TEA block of fast Cl channels. *(Filled symbols)* Mean values of fractional single channel current recorded from three separate channels. Error bars represent SEM. *(Inset, open symbols)* Values obtained from a single channel. *(Dotted lines)* Best fit of a single-site model (Eq. 6). *(Solid lines)* Best fit of a partial block model (Eq. 7) to the experimentally observed reduction in fractional current with TEA.

d caused by a time average of blocking and unblocking events. If this is the case, then the binding of TEA to the channel must lead to either a complete or partial occlusion of the permeation pathway. To attempt to differentiate between these two possibilities and to determine the approximate stoichiometry of the blocking reaction, kinetic reaction schemes were fit to the experimental dose-response measurements.

**Dose-response Measurements**

The relationship between fractional current magnitudes and TEA concentration is plotted in Fig. 2. For the simplest unimolecular blocking reaction scheme, where the binding of one TEA molecule to the channel results in a complete blockage of
current, the relationship between fractional current and blocker concentration is:

\[ \frac{I_B}{I_0} = \frac{K_D}{K_D + [\text{TEA}]}, \tag{6} \]

where \( I_B \) is the single-channel current amplitude measured in the presence of TEA, \( I_0 \) is the current measured in the absence of blocker, TEA is the TEA concentration bathing the formerly intracellular membrane surface, and \( K_D \) is the equilibrium dissociation constant for the binding of a single TEA ion to the blocking site. In Eq. 6 we specify that when TEA binds, the single channel conductance is 0, and that the reduced channel current is caused by the time averaging of rapid binding and unbinding. If, on the other hand, it is postulated that TEA binding only partially reduces single-channel current, the dose-response relationship is described by

\[ \frac{I_B}{I_0} = \frac{K_D + f[\text{TEA}]}{K_D + [\text{TEA}]}, \tag{7} \]

where \( f \) is the fractional current remaining when TEA is bound, and \( K_D \) is the equilibrium dissociation constant.

Increasing TEA concentration from 1 to 20 mM decreased the fraction of current remaining relative to the current with no blocker (Fig. 2), as predicted by both of the blocking reactions considered above. However, increasing [TEA]i concentration further, up to 250 mM, did not completely eliminate all ionic current. Although an adequate fit of the single-site complete block mechanism to the data collected in the presence of <50 mM [TEA]i could be obtained (not shown), the relationship between fractional current and TEA concentration could not be fit well by a simple single-site model (Fig. 2, dotted line) when all of the dose–response data were used. The single-site model predicted a much less steep dose–response curve than is observed. The best fitting parameter for the single-site model was \( K_D = 29.5 \pm 3.67 \) mM. A reasonable fit to both the low and the high concentration data could be obtained only with the blocking model where TEA binding did not lead to a complete reduction in single channel current (Eq. 7 and Fig. 2, dashed line). This partial block model successfully described the dose-response relationship at both low and high concentrations. The best fitting parameters for this blocking model were \( K_D = 15.3 \pm 0.57 \) mM, \( f \) (the fractional amplitude of the blocked open state) = 0.181 \pm 0.007. These parameters suggest that when TEA binds to the intracellular blocking site, the current through the channel is reduced to \( \sim 18\% \) of the unblocked current. It should be noted that the partial block model has no more free parameters than the single-site model, where it is implicitly assumed that the blocked single channel current is 0.

Amplitude Distribution Analysis

Typical amplitude distributions (with the amplitude of the normal distribution fit to the closed channel current subtracted) from an experiment on a patch containing several fast CI channels in the presence of 0–50 mM TEA are shown in Fig. 3 (left). Centered at 0 current in each distribution is a peak representing the current amplitude of the closed channel, and to the right of this peak are additional peaks representing the current when one, two, three, or four channels are open simultaneously. Because of the way in which these amplitude distributions were constructed (see Materials and Methods), no significance can be placed on the relative amplitudes...
or areas under the peaks representing the different numbers of channels in a patch. The reduction in peak amplitude, increased skewness, and changes in the width of the amplitude peaks is apparent as \( \text{T}_{\text{EA}} \) is increased. Also note how skewed to the left the amplitude distribution is, even in the absence of blocker. This is because of the abundance of short-shut events that are only partially resolved at the 6.4-kHz bandwidth of these experiments. Assuming that the frequency of these very short-shut events is not dependent on \( \text{T}_{\text{EA}} \) subsequent amplitude distribution analysis should not be affected by their presence. In this particular experiment, \( \text{T}_{\text{EA}} \) was increased sequentially in time from lowest to highest concentration. In other experiments (not shown), it was found that the measurable effects of \( \text{T}_{\text{EA}} \) block were

![Amplitude distributions obtained from the patch shown in Fig. 1 under control conditions and in the presence of increasing \( \text{T}_{\text{EA}} \).](image)

As is common with fast Cl channels, the number of active channels increased during the experiment. (Left) All points amplitude histograms comprised of similar numbers of digitized raw data points binned by their amplitude in 0.1-pA bins. Peaks in number of observations represent amplitude when (from left) zero, one, two, three, or four channels were simultaneously open. (Right) Amplitude distributions when a single Cl channel was active in the same patch, corrected for leakage current, and normalized so that the current amplitudes fall between 0 and 1, with 0 current representing the amplitude of the closed channel state and 1 representing the amplitude of the open channel state in the absence of blocker. The control amplitude distribution has been shifted to the left by 0.4 pA and is plotted on a separate x axis. (Dashed lines) Best fits of Eq. 1 convolved with the normal distribution fit to the closed channel amplitude distribution. Filtering, 6.44 kHz.

In Fig. 3 (right), we see the normalized amplitude distributions of the currents corresponding to the left-most open channel peak shown in Fig. 3 (left, solid lines) and, superimposed on these, the best fits of Eq. 1 convolved with the normal distribution fit to the closed channel amplitude distribution (dashed lines). The excellent fits follow the reduction in current amplitude and alterations of the shape of the distributions as \( \text{T}_{\text{EA}} \) is increased. The blocking \( (k_b) \) and unblocking \( (k_{-b}) \) rate constants for a simple two-state open channel blocking scheme are presented in Fig. 4. These results, from measurements in six separate experiments, showed that the unblocking rate constant was relatively independent of \( \text{T}_{\text{EA}} \) (range of 136,000 to 238,000 s\(^{-1}\) for \([\text{T}_{\text{EA}}]\) of 1 to 50 mM, respectively, with a mean value of 191,820 s\(^{-1}\)), while the blocking rate constant was a linear function of blocker
concentration (if the point at 50 mM TEA, where the small current amplitude makes the measurement error the worst, was excluded). A blocking rate constant of $16.24 \times 10^6$ M$^{-1}$s$^{-1}$ was calculated from these experiments. Dividing the unblocking rate constant by the blocking rate constant results in the $K_d$ for the blocking reaction. A value for the $K_d$ for TEA$i$ block of 11.8 mM was found using the rate constants calculated with the amplitude distribution approach. This value is about one-third the value for the blocking site in the poorly fitted single-site model (29.5 mM), but is similar to the value (15.3 mM) calculated for the partial block model.

*Lack of Voltage Dependence of TEA$i$ Block*

As shown in Fig. 5, currents through neuronal fast Cl channels were blocked by TEA$i$ at all membrane potentials between -60 and +50 mV. The current–voltage relation-
ship, plotted as the magnitude of the single-channel current against holding potential, under control conditions and in the presence of 25 mM TEA, is shown in Fig. 6. Current–voltage relationships of ≥12 CI channels from separate experiments exhibited nearly identical results. In contrast to the effects of external TEA application (Sanchez and Blatz, 1992), internally applied TEA seemed to block in a voltage-independent manner, suggesting that the blocking site was not located within the electrical field of the neuronal membrane. We have not systematically examined this lack of voltage dependence for TEA block in the presence of different permeant...
ion concentration gradients and, therefore, cannot exclude the possibility that the block is in fact voltage dependent, but that it is masked by interactions between permeant ions, TEA, and the channel, or that the voltage dependence of block by TEA, is really a current-dependent phenomenon, and that under the conditions of this study, there is no large inward K⁺ current (which is permeant, based on reversal potential shift measurements, through these channels) (Blatz, 1991) to oppose TEA⁺ from binding.

**Dwell Time Analysis of TEA⁻ Block**

Analysis of the distributions of the durations of open and closed dwell times gives insight into the nature of the kinetic mechanism underlying channel activity. In the following analysis, the mechanism underlying single-channel behavior is assumed to follow Markovian kinetics. One requirement of steady state dwell time analysis is that

![Stability plots of selected portions of dwell-time data used for subsequent analysis.](image)

**Figure 7.** Stability plots of selected portions of dwell-time data used for subsequent analysis. Intervals used in A were obtained in the absence of TEA and those used in B were obtained in the presence of 10 mM TEA. Mean open time of 50 sequential open intervals was calculated and this value was plotted against 50 on the x axis, and this process was repeated with nonoverlapping sequences of 50 intervals. Note that the y axes are linear (unlike the log axes of Blatz and Magleby [1986]). Filtering of 12.4 kHz.

the sampled kinetic activity must remain stable during the measurement period, and that the analysis must be restricted to one of the several "modes" of activity known for these channels (Blatz and Magleby, 1986b; Blatz, 1990). One measure of channel kinetic stability is the so-called "stability plot" (Blatz and Magleby, 1986b; McManus and Magleby, 1988; Weiss and Magleby, 1990). Fig. 7 shows stability plots for one typical fast CI channel under control conditions and in the presence of 10 mM TEA. For these plots, the mean of the first 50 open intervals was plotted against the open interval number, followed by the mean of the next 50 intervals plotted against the interval number, and so on, until the entire data set was plotted. The overall mean open duration increased from ~0.4–0.5 ms in this experiment. The long- and short-term variability of channel kinetic activity (Weiss and Magleby, 1990) was stable, either with or without TEA. The stability plots in Fig. 7 show only one brief period of anomalous behavior (solid bar). These events, from interval numbers ~15,000–
18,000 in the presence of 10 mM TEA, were excluded from subsequent analysis. Stability plots of mean closed interval durations were much more variable because of the wide variation in the durations of the closed intervals, and they were not as useful as the open intervals for determining channel kinetic stability.

Open and closed dwell time distributions for a representative single fast CI channel under control conditions (open symbols) and in the presence of 10 mM TEA (filled symbols) are presented in Fig. 8. The most-likely time constants and areas for the

![Figure 8](https://example.com/figure8.png)

**FIGURE 8.** Dwell-time distributions of open (A) and closed (B) interval durations recorded from a single fast CI channel held at $-47$ mV. (Filled symbols) Dwell time distributions obtained from single-channel currents recorded in the absence of TEA. (Open symbols) Dwell times obtained from currents recorded in the presence of 10 mM TEA. The best fit sums of exponentials to these distributions are represented as solid (control) and dashed (TEA) lines in the figure, and they are listed in Table I. C and D are the same experimental data as in A and B. Lines were calculated from simulations of scheme 1 taking into account the effects of filtering and noise. Filtering, 12.4 kHz.
unconditional fits of sums of exponential components to this data found by the method of maximum likelihood are shown in Table I. These maximum likelihood fits for each distribution considered separately are plotted as the solid (0 TEA) and dashed (10 TEA) lines in Fig. 8, A and B. The number of significant exponential components required to fit the open and closed interval distribution was the same under control conditions and in the presence of TEA. At the 12.4-kHz bandwidth used in these experiments and analyses, a single exponential component was required to fit the open interval distributions, and six components were required to fit the closed interval distributions. Performing the dwell time analysis with a lower frequency bandwidth, as in Blatz and Magleby (1986b) and Weiss and Magleby (1990), revealed a second significant open kinetic state with a true mean lifetime nearly identical to the true mean lifetime of the predominant open state (not shown). The following results are consistent with an equivalent blocking affinity of TEA for both of the open kinetic states.

| TABLE I |
| Time Constants and Relative Areas for the Exponential Components Fitted to the Unconditional Distributions of Open and Closed Interval Durations for a Representative Fast Cl Channel in the Absence and Presence of 10 mM TEA, |
| 0 TEA | 10 mM TEA |
| Open intervals | | |
| No. | τ (ms) | Area | τ (ms) | Area |
| 1 | 0.380 ± 0.12 | (1.00) | 0.527 ± .23 | (1.00) |
| Closed intervals | | |
| No. | τ (ms) | Area | τ (ms) | Area |
| 1 | 0.025 ± 0.008 | (0.762) ± 0.02 | 0.021 ± 0.007 | (0.725) ± 0.01 |
| 2 | 0.212 ± 0.03 | (0.030) ± 0.008 | 0.151 ± 0.054 | (0.048) ± 0.02 |
| 3 | 2.77 ± 1.2 | (0.075) ± 0.019 | 1.82 ± 1.03 | (0.104) ± 0.05 |
| 4 | 15.79 ± 3.2 | (0.078) ± 0.009 | 10.11 ± 7.65 | (0.090) ± 0.03 |
| 5 | 39.79 ± 8.3 | (0.041) ± 0.014 | 16.024 ± 10.1 | (0.024) ± 0.01 |
| 6 | 441.35 ± 65.7 | (0.013) ± 0.015 | 326.14 ± 97.5 | (0.009) ± 0.006 |

Values reported are means of three single channels ± SEM.

The most consistent effect of TEA on the unconditional distributions was an increase in the time constant of the open kinetic state. This is what would be expected if TEA binds to the channel when it is in the open state, and that the transitions between the open and the blocked state are much faster than can be resolved. This result does not rule out additional binding steps between one or more of the closed channel kinetic states, but it suggests that the predominant binding reaction is between the open kinetic state and a blocked state that must reenter the open state before channel closing. One would not expect that the addition of a such a very fast channel blocker would increase the number of detected open or shut states, since the lifetimes of the open and blocked states are time averaged into a compound open state of reduced single-channel conductance. The closed interval distributions were less affected by TEA, although a small decrease in the relative area of the fastest shut component and an increase in the area of the next fastest component was often observed.
Kinetic models were examined for their ability to predict these distributions. As expected, the distributions of unconditional open and closed interval durations could be predicted exactly by a variety of kinetic reaction schemes when the control and blocked channel data were considered separately. A more discriminating approach is to simultaneously fit the experimental distributions collected in the presence of several blocker concentrations with a single kinetic scheme where blocked states are added to the basic model. The simplest model that could reasonably account for the TEA block was

\[ C_8 \rightarrow C_7 \rightarrow C_6 \rightarrow C_5 \rightarrow C_4 \rightarrow C_3 \rightarrow [O_1 \rightarrow OB_2] \] (8)

with rate constants shown in Table II. In this scheme, all of the transitions between the open state, \( O \), and the blocked state, \( B \), were assumed to be rapid enough so that when the channel entered the open state from the terminal closed kinetic state, it immediately fluctuated between the open and blocked states with all transitions between the open and the blocked state, resulting in true events with durations less than that required to produce observed open or closed events. The result of this would be observed transitions between a fully closed state and a time-averaged open state of reduced conductance. The single-channel current amplitude would reflect the amount of time spent in the open state relative to the blocked state, and would, therefore, decrease with increasing blocker concentration. The solid lines in Fig. 8, C and D, are the predictions of this simple kinetic model using rate constants that were found by simultaneously fitting the open and closed distributions in the absence of blocker and in the presence of 10 mM TEA. The increase in the time constant of the open interval distribution was fairly well fit by this simple kinetic model, except that the kinetic model predicts a larger increase in mean open duration than is observed.

### Table II

| Rate constant | Value ± SEM |
|---------------|-------------|
| \( k_{12} \) | \( 16.24 \times 10^6 \) |
| \( k_{21} \) | 191,820* |
| \( k_{13} \) | 5,853 ± 356 |
| \( k_{31} \) | 35,517 ± 12,000 |
| \( k_{54} \) | 10,710 ± 2,450 |
| \( k_{43} \) | 1,109 ± 249 |
| \( k_{43} \) | 3,252 ± 127 |
| \( k_{54} \) | 3,252 ± 127 |
| \( k_{56} \) | 3,252 ± 127 |
| \( k_{65} \) | 3,252 ± 127 |
| \( k_{76} \) | 3,252 ± 127 |
| \( k_{78} \) | 3,252 ± 127 |

Values for rate constants are in s\(^{-1}\), except for the on rate, which is in s\(^{-1}\) \cdot M\(^{-1}\). Values are mean ± SEM (n = 3). No errors are shown for the on and off rate constants (\( * \) and \( \dagger \)) because they were constrained so that the \( K_D \) for the blocking reaction was 11.8 mM.
The model derived from simultaneous fitting did not predict the changes in the distributions of closed intervals very well. This suggests that besides binding to the channel when in the open kinetic state, TEA probably binds to the channel when it is closed. Alternatively, more complicated kinetic models may be required. Two other separate Cl channels were analyzed using these methods, and similar results were obtained. Because the number of closed kinetic states did not increase with TEA, there is no compelling justification to extend the kinetic modeling to include additional blocked shut states. This simplistic model would not be expected to predict much of an effect on the closed interval distributions because (a) only a single open state was used, and (b) TEA is allowed to bind only to the open kinetic state. The relatively poor fit of the model to the observed closed intervals (particularly at long durations) may also be caused by the small number of long-closed events.

**DISCUSSION**

This study describes the effects of internally applied TEA on the fast chloride channel in neurons. The kinetic properties of TEAi block of these Cl channels was complex, but a major blocking reaction occurred between the blocker and the open kinetic state, such that when blocked, the channel must pass through the open state to close. The block appeared as a reduction in channel conductance because of the time averaging of extremely fast blocking and unblocking transitions. The apparent mean lifetime of the open channel kinetic state increased with TEA in a manner consistent with this open state blocking model. Blockade of the open kinetic state should not be confused with the common notion of a blocking molecule actually binding inside the pore of the open-ion channel. The experiments in this study do not directly address this issue, except that the apparent lack of voltage dependence of the block is weak negative evidence against blocking within the channel pore. The results of this study are limited only to the kinetic states of fast Cl channels.

It was possible to analyze dose–response relationships, amplitude distributions, and dwell time data from individual Cl channels at different [TEA]. All three of these analytical methods gave similar results that are consistent with a rapid blocking reaction between TEA and the open kinetic state. The inability of the simplest open state blocking models to predict the complicated changes observed for the distributions of closed intervals suggest that TEA must also interact with one or more of the channel's closed kinetic states. The consistent reduction in the percent of time that the channel is in the open state with TEA also argues for blocking reactions between the closed channel states and TEA.

An interesting property of TEA block of these channels was that the dose–response relationship indicated that when TEA was bound, the single-channel conductance was not reduced to 0, but to a partially conducting value. Since all of the relevant reactions are so rapid as to be mostly unresolvable, it would not be expected that the partial conducting blocked state could be directly observed at the resolutions of the present study. A possible mechanism for this observation could be that, when TEA binds, the channel pore is not completely functionally occluded, but it is left partially open so that steric and/or electrostatic interactions reduce the throughput of the pore. Alternatively, additional binding reactions could be occurring between TEA and the channel that are even faster than those observed in the kinetic experiments.
reported here. These more rapid reactions, even if occurring between fully open and fully blocked conductive states, would be manifested as partially conducting states.

Comparison of TEA Block of Neuronal Fast Cl Channels with Other Cl Channels

Other Cl channels are also blocked by millimolar concentrations of TEA. Lukács and Moczydlowski (1990) described a Cl channel isolated from lobster nerve and inserted into planar lipid bilayers. This channel was blocked by a factor of 40–60% in the presence of 100 mM TEA. Unlike neuronal fast Cl channels, the lobster channel was sensitive to TEA from only one membrane surface, although it is unknown which surface it was because of experimental limitations. TEA block of the lobster Cl channel appears quite similar to that of neuronal fast Cl channels in that the major effect is a reduction of single-channel current amplitude, presumably because of time averaging of rapid on and off kinetics. Other than the reduction of conductance, Lukács and Moczydlowski (1990) observed no alterations of single-channel kinetic behavior. The increase in mean open lifetime observed in our experiments would probably not have been resolved in the experiments on the lobster channel because of the necessary bandwidth limitations of bilayer recording. An anion-selective channel from human platelet surface membrane seems also to be blocked by TEA (Manning and Williams, 1989).

Similarities of TEA Block of Cl and K Channels

The effects of block by internal TEA are similar to those of TEA block of several kinds of K channels (Stanfield, 1983). Most voltage-sensitive K channels are blocked to some extent by TEA (Stanfield, 1983; Hille, 1992). Block by TEA of K channels can be roughly divided into two types. One type of TEA block, of which the classic example is internal block of the delayed rectifier K channel in squid giant axon and frog node of Ranvier (Armstrong and Binstock, 1965; Armstrong, 1969; Armstrong and Hille, 1972; French and Shokimas, 1981; Swenson, 1981), is usually voltage dependent, and increasing the alkyl chain length, either symmetrically or asymmetrically increases the affinity of the drug for the channel. At other blocking sites, such as the external node of Ranvier delayed rectifier K channel, TEA block is often not voltage dependent and increasing the hydrocarbon portions of TEA reduces, rather than increases the drug’s affinity. TEA block of neuronal fast Cl channels does not fit neatly into either of these categories. External TEA block is voltage dependent (Sanchez and Blatz, 1992), much like internal TEA block of delayed rectifier K channels, large-conductance Ca-activated K channels (BK channels) (Blatz and Magleby, 1984; Yellen, 1984; Villarroel et al., 1988; Carl et al., 1993), and cloned Shaker channels (Kirsch et al., 1991; Taglialatela et al., 1991; Choi et al., 1993). Unlike delayed rectifier channels, the fast Cl channel is blocked with higher affinity by external quaternary ammonium ions with longer symmetric alkyl chains applied either internally or externally (Sanchez and Blatz, 1991). Internal TEA blocks fast Cl channels with much less or no voltage dependence, as shown in the present study, but it is also blocked at higher affinity by longer chain quaternary ammonium compounds (Sanchez and Blatz, 1991).

The kinetics of TEA block of K-selective ion channels can be extremely rapid, with large on and off rates (Stanfield, 1983). These rapid blocking and unblocking
transitions lead to an apparent reductions in single-channel current amplitude with TEA, as occurs with internal application of TEA to BK channels (Blatz and Magleby, 1984; Villarroel et al., 1988; Carl et al., 1993). TEA block exhibits slower kinetics with external application to BK channels (Langton et al., 1991; Carl et al., 1993) and internal block of several cloned Shaker K channels (Kirsch et al., 1991; Taglialatela et al., 1991). In the present study, we have demonstrated that internal application of TEA results in a rapid block of neuronal Cl channels, placing the blocking site in a category similar to the internal site of BK channels.

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