BLOCKAGE OF SQUID AXON POTASSIUM CONDUCTANCE BY INTERNAL TETRA-*N*-ALKYLAMMONIUM IONS OF VARIOUS SIZES

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ABSTRACT We have studied the effects of the tetra-*n*-alkylammonium (TAA) ions, $(C_nH_{2n+1})_4N^+$, $n = 1$–6, on the potassium conductance of voltage-clamped squid giant axons. Studies using tetrahexylammonium were not quantitatively analyzed as its effect was insufficiently reversible. Each in this series of symmetric ions of graded size blocks the potassium conductance when added to the internal perfusion fluid. There is a general trend for blocking potency to increase with increasing size. We attribute this to stronger interactions of the longer alkyl side chains with hydrophobic regions of the membrane near the channels. Steady-state block by the TAA ions, $n = 2$–5, showed identical voltage dependence, apparently sensing about 15% of the transmembrane voltage, and kinetics of block onset were qualitatively similar. We conclude that the site of action for these ions is the same. Block by TMA is about twice as steeply dependent on voltage. In its action, TMA resembles the alkali cations (French et al., 1979. *Biophys. J.* 25(2, pt. 2):307*a*) more than the larger TAA ions. Our results suggest that access to the inner mouth of the K channel is even less restricted than has been previously thought. A calculation indicates that the lumen of the channel cannot be both wide enough to admit the TAA ions and long enough to account for the voltage dependence of block. We consider possible ways to resolve this paradox.

INTRODUCTION

Tasaki and Hagiwara (1957) reported that tetraethylammonium (TEA) ions when injected into squid giant axons caused a prolonged action potential and eliminated most of the late current under voltage clamp conditions. The results were confirmed by Armstrong and Binstock (1965), who concluded that the prolongation of the action potential was due to TEA's interference with the outward potassium current. Armstrong and Binstock also showed that, with an external medium of high potassium concentration, TEA blocked outward potassium currents more strongly than inward currents causing an "anomalous rectification." In subsequent papers, Armstrong (1966*a*, 1969, and 1971) elucidated the kinetics of the action of TEA and several related quaternary ammonium (QA) compounds. This work led to the view that the QA ions were able to enter and occlude the inner, relatively nonselective mouth of the potassium channel after the channel's voltage-dependent "gate" had opened. The QA ions were presumed, because of their large size, to be unable to pass through a more

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selective "tunnel" region of the channel. Evidence for these views is summarized by Armstrong (1975 *a*, and *b*).

In his papers of 1969 and 1971, Armstrong showed that a number of ions, having the general formula $(C_2H_5)_3N^+$—R, blocked the potassium conductance in a manner qualitatively resembling the action of TEA. R groups consisted of *n*-alkyl chains up to a maximum of C_{12}, and phenyl-alkyl groups with the alkyl chain ranging from C_1 to C_3. Generally, the blocking potency increased with increasing size, and consequent increasing hydrophobicity, of the R group. In contrast, octyltrimethylammonium at 1.2 mM produced no detectable inactivation of the potassium conductance. These observations led Armstrong to suggest that the blocking potency was dependent on a hydrophobic interaction between the R group and the membrane, while specificity for the K conductance was due to the three ethyl groups surrounding the nitrogen atom. The near equality of the radii of a TEA and a K ion with a single hydration shell adds credence to this suggestion.

Two published observations, however, suggest that access to the innermost blocking site of the potassium channel may not be limited to ions having charged head groups with a diameter of 8 Å or less. First, Armstrong (1966*b*) noted in passing that tetra-*n*-propylammonium bromide caused a reduction of both sodium and potassium currents. Second, strychnine and its quarternary derivative *N*-methylstrychnine applied internally to frog node of Ranvier causes a K-conductance block (Shapiro, 1977) very similar to that produced by other QA ions (Armstrong and Hille, 1972).

We describe experiments to investigate the effect of a series of symmetric quaternary ammonium ions on the potassium conductance. This work was undertaken in the hope of firmly establishing a limiting size above which a symmetric ion would not block the K channel. Such an ion would be a valuable tool, providing an inert substitute to maintain ionic strength in other experiments which demand variation of internal concentrations of ions that pass through or interact with the K channels. Although we have studied the effect of tetraalkylammonium ions for alkyl chain lengths of 1–6 carbon atoms, no "inert" ion was found. Each species tested caused a profound reduction in the potassium current. Brief reports including some of these data have been presented (Shoukimas and French, 1979; French and Shoukimas, 1979; French et al., 1979).

METHODS

Experimental Preparation

The experiments were performed using internally perfused, voltage-clamped squid giant axons. Squid were provided by the Marine Biological Laboratory, Woods Hole, Mass. In general, methods were similar to those used in earlier studies (French and Wells, 1977). We used a conventional axial wire voltage clamp, compensating for 2 Ω cm^2 of series resistance. Settling time for the current trace in response to a hyperpolarizing voltage step was <20 μs. Voltage, $E = V_{inside} - V_{outside}$, was measured between internal and external glass pipettes, with tip diameters of 50–80 μm, filled with 0.5 M kCl in 1–2% agar. A floating platinum wire shunt in the pipettes reduced the high frequency impedance (Fishman, 1973). The axial wire, fixed concentrically within the perfusion cannula, and the internal voltage sensing electrode entered the axon from opposite ends of the axon. We initiated perfusion, without the use of enzymes, by passing the cannula completely through the axon, one or more times, using suction to remove the axoplasm.

Temperature of external solutions entering the chamber was maintained at 8 ± 0.1°C during data collection. External solution flowed over the axon at ~3 ml min^{-1} (chamber volume ~0.05 ml). Between test pulses, the membrane potential was held at $E = -80$ mV. In all experiments the sodium conductance was suppressed by including 0.5 or 1 μM tetrodotoxin (TTX) in the external solution.

Data Acquisition

We used a Digital Equipment Corp. (Marlboro, Mass.) PDP-11/10 computer to control a custom interface which delivered voltage clamp command pulses and accepted the analog current record after low pass filtering (−3 dB at 80 kHz) by a Krohn-Hite 3202R eight-pole filter (Krohn-Hite Corp., Avon, Mass.). An Analog Devices DAC-10DF ten-bit digital-to-analog converter (Analog Devices, Inc., Norwood, Mass.) generated the voltage pulses. Incoming data were accepted using an Analog Devices SHA-2 sample-and-hold amplifier and an Analog Devices ADC-1103, ten-bit analog-to-digital converter. The digitized signal was taken in via direct memory access and the record then written out to a nine-track magnetic tape before delivery of the next command pulse sequence. In these experiments, current was usually sampled at 50-μs intervals.

Correction for linear leakage and capacitative components of the current was performed off-line by adding three successive current records obtained in response to command voltages V, $-V/2$, and $-V/2$ from the holding potential where V represents a sequence of up to three contiguous pulses. A rest period of ~5 s was allowed between each pulse sequence, so that at least 15 s elapsed between consecutive test pulses to levels positive with respect to the holding potential. This avoided any appearance of frequency dependent block in the successive pulses used to collect a family of records to determine current- (and conductance-) voltage relations.

All of the data shown and analyzed represent records corrected for leakage and capacitative components as described.

Conductances

In experiments using tetramethylammonium (TMA), the chord conductance, g_c, was estimated for each point on the instantaneous I-E relation according to the expression $g_c(E) = I(E)/(E - E_K)$, where E_K is the reversal potential (see Fig. 2). To compare the actions of TEA and the larger tetraalkylammonium ions, the conductance was estimated from the step in current that occurred when the voltage was returned to the holding potential at the end of a test pulse. Thus, $g(E) = \Delta I/\Delta E$, where $\Delta I = I(E) - I(-80)$, and $\Delta E = E - (-80)$. When the instantaneous I-E relation is perfectly linear, this is equivalent to a determination of the chord conductance, g_c, at the end of the test pulse. The single step estimate of conductance provided an adequate basis for comparison of the effects of TEA and the larger ions because the instantaneous I-E relations were close to linear under the experimental conditions. These, and other large quaternary ammonium ions (Armstrong, 1971, Fig. 3 *c*), equilibrate too slowly to cause nonlinearities in the instantaneous I-E relation. The only observed nonlinearity was slight rectification due to inequality of potassium concentrations at the internal and external surfaces of the membrane. This effect was minimal, since $E_K \sim 0$ at the end of test pulses near the middle of the voltage range considered. A worst case indication of the quantitative effect of basing the analysis on g, instead of g_c, was obtained by analyzing data from three experiments on TEA in terms of chord conductance, g_c. Data for $E = 0$, 60, 120 mV, and [TEA] = 0, 0.1, 1.0, 10.0 mM were used. Parameter values indicating voltage dependence and relative binding affinity for TEA were $s = 0.14$ and K = 0.5 mM based on g_c (cf. Table V, $s = 0.14$ and K = 0.36 mM based on g). For the overall analysis, g was used since many fewer pulses were required to obtain data over a wide range.

Analysis

Data recovery and much of the analysis and curve-fitting were performed with the PDP-11/10 computer used to run the experiments. Some curve-fitting and graphics were also done using the MLAB (Knott and Reese, 1972; Knott and Shrager, 1972) program implemented on the DECSYSTEM-10 at the Division of Computing Research and Technology of the National Institutes of Health, Bethesda, Md.

TABLE I
SOLUTION COMPOSITIONS (CONCENTRATIONS IN MILLIMOLAR)

Solution	Na^+	K^+	TAA^+	$Tris^+$	Mg^{2+}	Ca^{2+}	Cl^-	F^-	glutamate$^-$	HPO_4^{2-}	Sucrose	pH*
TTX-ASW	430	10	—	10	50	10	570	—	—	—	—	7.0
xTTA, 300K	—	300	x	—	—	—	x	50	200	25	(505-2x)	7.2

*Adjusted at room temperature, ~22°C.

Solutions

Table I gives the composition of the various internal and external solutions used. In experiments analyzed in this paper the following tetraalkylammonium (TAA) salts were used: tetramethyl-, TMA; tetraethyl-, TEA; tetrapropyl-, TPrA; tetrabutyl-, TBA; tetrapentyl-, TPeA. A few experiments have also been carried out using tetrahexylammonium (THxA). All of the TAA ions were obtained as the chloride salts from Eastman Organic Chemicals, Rochester, N.Y. The supplier specified the limits of purity for all except TBA as ≥98%, with ≤2% water. For the TBA-Cl sample, purity was specified to be ≥90%. All of these salts are quite hygroscopic, with TBA-Cl being the extreme case. Consequently, care was taken to minimize the exposure to air when samples were weighted out to make stock solutions.

We did not have available the necessary facilities to routinely purify all salts used. Procedures required to obtain highly pure crystals of the chloride salts of the larger tetraalkylammonium ions are particularly difficult (see, for example, Unni et al., 1963, and Kay et al., 1965). For any further studies the bromide salts may prove to be a more convenient choice.

For TPrA-Cl and TBA-Cl we performed experiments using recrystallized samples as well as using the salts directly as supplied by Eastman. The TPrA-Cl was twice recrystallized from ethanol solution by adding ethyl acetate, giving a clean, white crystalline precipitate. In the case of TBA-Cl, the salt was dissolved in hot benzene and precipitated by the addition of hot hexane. This procedure was not totally satisfying as the precipitate had an oily appearance and very rapidly absorbed water from the air. In neither case were we able to detect any difference in the effect on the potassium conductance between the recrystallized and the commercial samples. We thus have no evidence that the physiological effects are due to small amounts of impurity in the salts used.

Junction Potentials

Junction potentials, measured at $(V_i - V_o)$ between internal and external solutions connected by a salt bridge of 3 M KCl in 3% agar, using the experimental electrodes were ~3 mV. There was little variation between controls and the TAA-containing solutions, since the TAA salts, other than TMA, were present at relatively low concentrations. Corrections for junction potentials have been made only in the data, from experiments with TMA, presented in Fig. 2.

EXPERIMENTAL RESULTS AND ANALYSIS

Internal TMA Causes Voltage-Dependent Block of K Channels

Although considerably less potent than the larger TAA ions, TMA at sufficiently high internal concentration produces a strong block of the excitable potassium conductance. The block is voltage-dependent, and with 100 mM TMA occurred too rapidly for us to resolve any time-course of onset after a step change in voltage. In Fig. 1 (upper trace) we show that the block is favored by positive transmembrane voltages to such an extent that a step, from a prepulse voltage of E = 60 mV to E = 180 mV, causes an instantaneous decrease in the outward current. Furthermore, we did not observe any biphasic, "hooked" tailed currents (e.g., Fig. 1, lower trace) on repolarization (cf. Armstrong, 1966, 1969; Yeh and Narahashi,

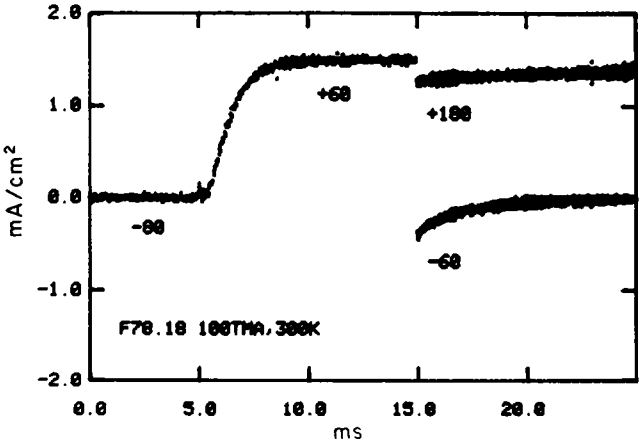

FIGURE 1 Two superposed voltage clamp current records showing, on the upper trace, an instantaneous decrease in current caused by internal tetramethylammonium ions and following a positive step in transmembrane voltage, and on the lower trace, the monotonic decline of inward tail currents toward zero after a step to $E = -60$ mV. Linear leakage and capacitative currents have been subtracted. Sample points in the second pulse (upper, $E = +180$ mV; lower, $E = -60$ mV) start 40 μs after the beginning of the voltage step, and continue at 10 μs intervals. External solution, TTX-ASW; internal solution contained 100 mM TMA, 300 mM K (full details in Methods); temperature 8°C; transmembrane voltage E, in millivolts, is shown alongside each portion of the records.

1977; Cahalan, 1978). Consequently, TMA appears to enter and leave the open channels very rapidly after steps in the transmembrane voltage.

Fig. 2 shows the instantaneous (60 μs) current-voltage relation (upper), and the corresponding chord conductances (lower) determined from the experimental run that provided the records of Fig. 1. The more negative reversal potential measured in the presence of TMA is due to the reduction of periaxonal potassium accumulation during the prepulse that activated the conductance. TMA significantly reduced the prepulse current (cf. Bezanilla and Armstrong, 1972). We shall show below that the dependence on voltage of the K conductance block by the larger TAA ions is only about half as steep as it is for TMA. In potency, voltage dependence and rapidity of action, TMA more closely resembles the alkali cations Cs, Na, and Li (Bezanilla and Armstrong, 1972; French and Wells, 1977) than the larger TAA ions. For this reason we reserve a more detailed analysis of the effects of TMA and functionally similar ions for a separate paper (French et al., 1979; and in preparation).

TAA ions, $(C_nH_{2n+1})_4N^+$, for n = 2–6, All Produce a Time-dependent Block of Potassium Currents

At appropriate concentrations and sufficiently positive voltages, all of these compounds examined produced "inactivating" potassium current records similar to those described by Armstrong (1969 and 1971) for TEA and its R-triethylammonium derivatives. Families of voltage clamp current traces from experiments using TEA and TPeA are shown in Fig. 3. At concentrations necessary to produce a particular level of steady-state block, the rate of block is slower for the larger members of the series, TBA, TPeA, and THxA, than for the smaller members, TEA and TPrA. However, as illustrated in Fig. 3, the larger ions attain a greater

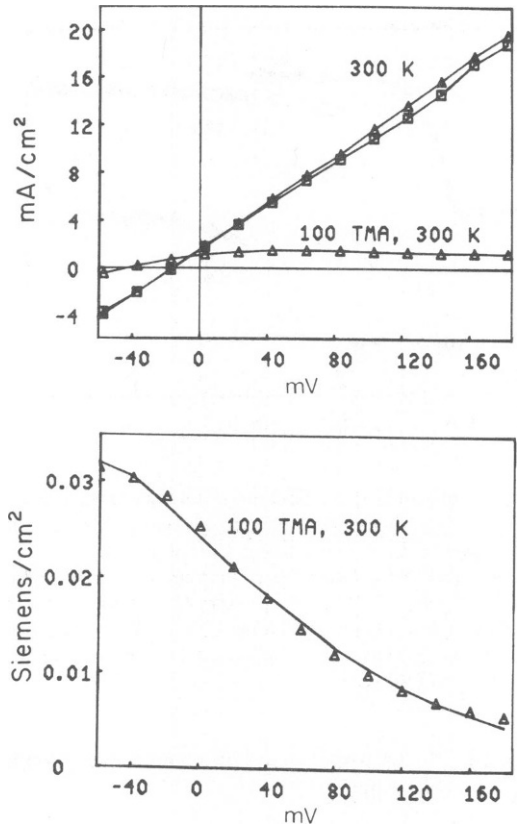

FIGURE 2 Voltage-dependent block of the potassium conductance by internal tetramethylammonium ions. Internal solution for all runs contained 300 mM K. Same axon as for Fig. 1. Upper: Instantaneous current-voltage data, determined at 60μs after a 10-ms prepulse, to $E = +60$ mV, used to activate the K conductance. The control runs were done before and after the axon was perfused with the solution containing TMA. Lower: Chord conductances (g_c, as defined in Methods) calculated from the instantaneous I-E data obtained with TMA present. Conductances in control runs were approximately constant at ~0.095 s cm^{-2}. The line joins points derived from the control conductances using the expression, $g' = 0.54\, g/[1 + 0.3(E + 11)F/RT]$, where g' approximates the conductance in the presence of TMA and g is the mean of control conductances before and after the TMA run.

degree of block in the steady state than do the smaller ones at the same, or even higher, concentration.

Behavior upon repolarization with internal TAA ions is also similar to that observed by Armstrong using the R-triethylammonium ions. Inward tail currents after a depolarizing pulse may be nonmonotonic, increasing briefly before declining toward zero. This is clearly seen for TEA in Fig. 3. Slower, but qualitatively similar kinetics are discernible in some of the TPeA records in Figs. 3 and 5. It thus appears that unblocking precedes the time and voltage dependent decay of the conductance.

From a cursory examination of the records in Fig. 3 it appears that the rate of "inactivation" of the potassium currents produced by the tetraalkylammonium ions is little, if at all, affected by voltage. A more careful scrutiny shows that the decline of the current is

slightly, but visibly, speeded up at the highest voltages. This effect is emphasized in Fig. 4 by the crossing of superposed current records taken during pulses to $E = 40$ and 120 mV using an axon perfused with a solution containing 0.5 mM TBA.

In Fig. 5 we superpose records taken at a constant voltage with different internal concentrations of TPeA. Two points become clear: first, the rate of block onset is increased as the concentration of the blocking ion increases, and second, that the initial rate of rise of the potassium current after a depolarizing voltage step is not noticeably changed by the presence of the TAA ions.

Dependence of the Rate of Block Onset on TAA Concentration and Voltage

Two methods were used to quantify the rate at which the block of the potassium conductance approached a steady state. When the decline of the currents, in a family of records, largely occurred after the time at which control currents had reached a maximum value, the declining phase, after this time, was fitted with a single exponential and the time constant, τ, determined (see Fig. 6). When, as was true for the higher concentrations of TEA and TPrA, the block was largely complete by the time required for control currents to reach a maximum, we calculated the ratio, $I_{K+X}(t)/I_K(t)$, point by point for records taken in the presence (i_{K+X}),

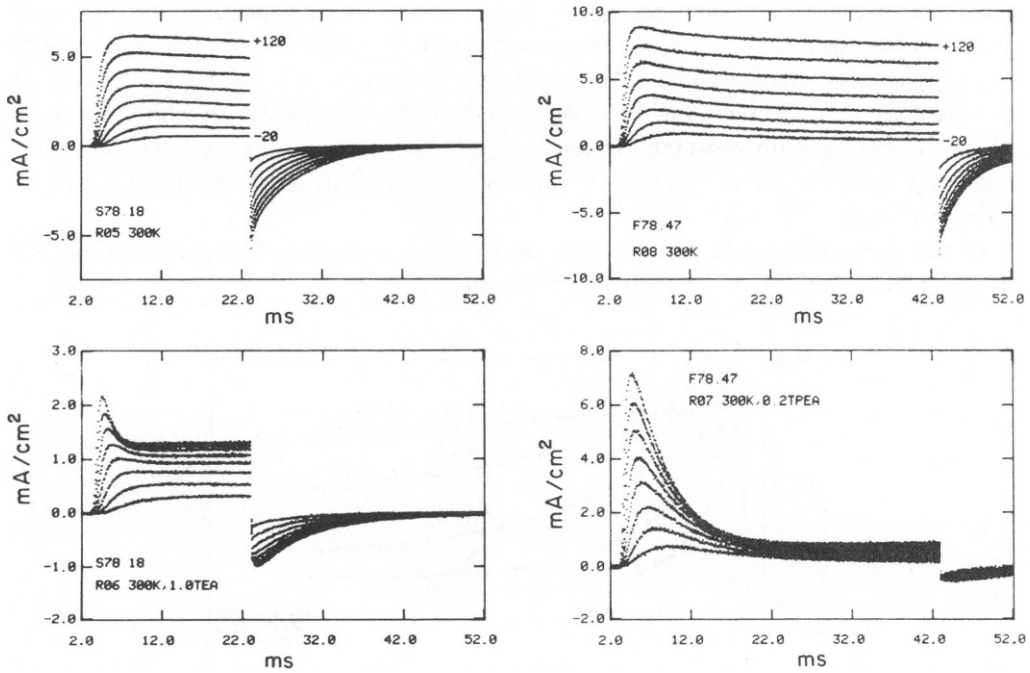

FIGURE 3 Voltage clamp current records for pulses to $E = -20, 0, 20 \ldots 120$ mV from a holding potential of $E = -80$ mV. Linear leakage and capacitative components of the current have been subtracted. In each case, external solution was TTX-ASW, temperature = 8°C. Upper left: internal solution, 300 mM K (immediately before TEA). Lower left: internal solution, 1.0 mM tetraethylammonium, 300 mM K. Upper right: internal solution, 300 mM K (Immediately after TPeA). Lower right: internal solution, 0.2 mM tetrapentylammonium, 300 mM K. Data in pairs of frames on left and right are from two separate experiments.

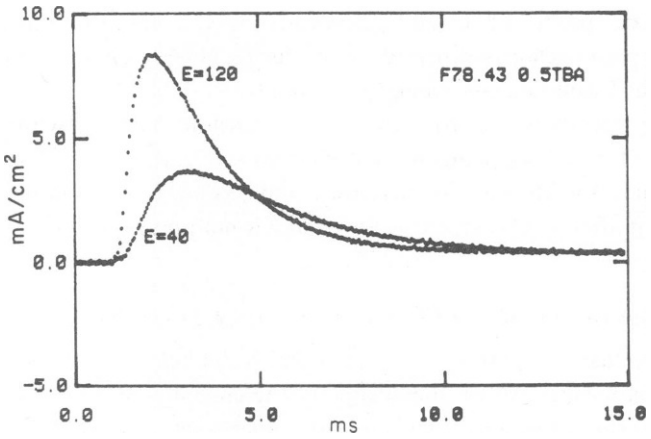

FIGURE 4 Voltage-dependent rate of block of the K conductance by internal tetrabutylammonium indicated by the crossing of voltage clamp current records taken at E = 40 and 120 mV. Linear leakage and capacitative currents have been subtracted. External solution, TTX-ASW; internal solution contained 0.5 mM TBA, 300 mM K; temperature 8°C.

and in the absence (I_K), of the blocking ion. After a brief period in which the currents were too small to obtain a reliable estimate, the ratio declined exponentially towards a steady-state value. Again, the time constant, τ, for the decline was determined (see Fig. 7). Clearly, the two methods are equivalent when the decline occurs during a period in which the control current, I_K, is essentially constant. We routinely estimated values of τ for block onset by TEA and TPrA using the ratio $I_{K+X}(t)/I_K(t)$, and for block onset by TBA and TPeA by directly fitting the decline of $I_{K+X}(t)$.

Typical values of τ for the various TAA ions studied are shown in Table II, as a function of voltage, and in Table III, as a function of TAA concentration.

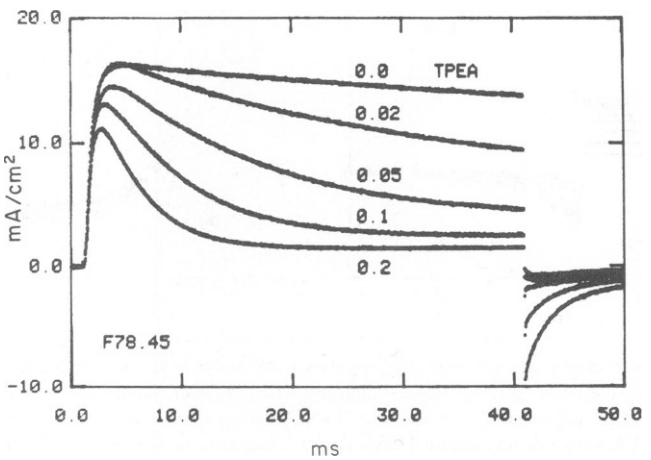

FIGURE 5 Records of K channel currents obtained under voltage clamp to E = 120 mV in the presence of various internal concentrations of tetrapentylammonium. External solution, TTX-ASW; internal solution contained 300 mM K plus the concentrations (mM) of TPeA indicated on the figure; temperature = 8°C.

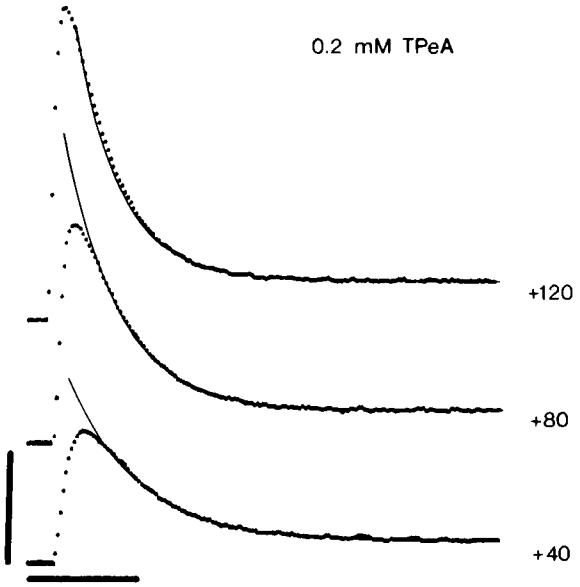

FIGURE 6 Three records from an experiment with 0.2 mM tetrapentylammonium inside the axon. Other conditions as for Fig. 5. The declining phase of the current in each record was approximated by a single exponential. The thin lines indicate nonlinear least squares fits to the data points. Time constants for decline were: 6.2 ms, E = 40 mV; 4.7 ms, E = 80 mV; 2.9 ms, E = 120 mV. Scale bars: vertical, 4 mA cm^{-2}; horizontal, 10 ms. Voltage indicated alongside each record. Experiment F78.45.

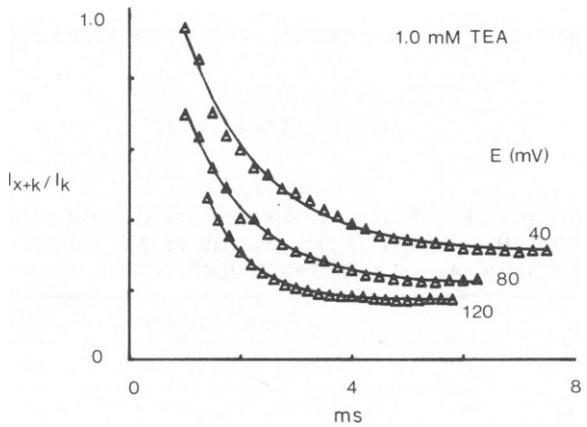

FIGURE 7 Time-courses of decline of the point-by-point ratio of current with 1.0 mM tetraethylammonium to control values observed in its absence. The continuous lines are nonlinear least squares single exponential fits to the data points with the time constants: 1.4 ms, E = 40 mV; 1.2 ms, E = 80 mV; 0.8 ms, E = 120 mV. Both internal solutions contained 300 mM K. External solution, TTX-ASW. Temperature 8°C. Experiment S78.16.

TABLE II

REPRESENTATIVE VALUES OF THE TIME CONSTANTS, τ ms, FOR THE DECLINE OF CURRENT, I_{K+X}, OR CURRENT RATIO, I_{K+X}/I_K, CAUSED BY THE PRESENCE OF INTERNAL TETRAALKYLAMMONIUM IONS AT SEVERAL VOLTAGES

TAA species	Exp. No.	E – mV				
		40	60	80	100	120
(mM)						
TEA,* 1.0	S78.16	1.4	1.7	1.2	1.0	0.8
TPrA,* 1.0	F78.34	1.8	1.2	1.1	0.9	0.9
TBA,‡ 0.5	F78.43	3.3	2.7	2.3	2.0	1.7
TPeA,‡ 0.2	F78.45	6.2	5.2	4.7	4.2	3.9

*τ determined from the decline of the ratio of currents, $I_{K+X}(t)/I_K(t)$.
‡τ determined from the decline of single current records, $I_{K+X}(t)$.

For all ionic species and voltages analyzed when the [TAA] < 1 mM (11 cases from six different axons), the rate of block onset was linearly dependent on TAA concentration. Thus,

$$1/\tau(E) = a(E)[TAA] + b(E). \quad (1)$$

Fig. 6 illustrates this point with data taken at four different voltages using TPeA as the blocking ion.

The dependence of $1/\tau$ on E is more complex. At higher concentrations, increasing E clearly increases $1/\tau$ (see Fig. 6 and Table II). In contrast, when [TPeA] = 0.05, $1/\tau$ was virtually independent of voltage (Fig. 6). In other cases, at low concentrations, small decreases in $1/\tau$ for increasing voltage were seen in the experimental data. This behavior is implied by the lines drawn in Fig. 8. We consider two limiting cases. When the [TAA] is very small, Eq. 1 becomes

$$1/\tau(E) \sim b(E), \quad (2)$$

and the voltage dependence of $1/\tau$ represents that of $b(E)$. At very large values of [TAA], we have

$$1/\tau(E) \sim a(E)[TAA], \quad (3)$$

TABLE III

REPRESENTATIVE VALUES OF THE TIME CONSTANTS, τ ms, FOR THE DECLINE OF CURRENT, I_{K+X}, OR CURRENT RATIO, I_{K+X}/I_K, IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF TETRAALKYLAMMONIUM IONS AT E = 120 mV

TAA species	Exp. No.	TAA Concentration τ in millimolar						
		0.05	0.1	0.2	0.3	0.5	1.0	3.0
TEA*	S78.18	—	3.0	—	1.9	—	1.1	0.8
TPrA*	F78.34	—	3.8	—	2.2	—	0.9	0.5
TBA‡	F78.43	14.6	7.8	4.6	—	1.7	—	—
TPeA‡	F78.45	14.2	7.2	3.9	—	—	—	—

*τ determined from the decline of the ratio $I_{K+X}(t)/I_K(t)$.
‡τ determined from the decline of single current records, $I_{K+X}(t)$.

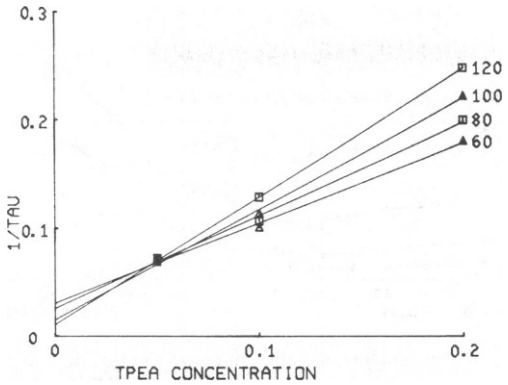

FIGURE 8 The rate of decline ($1/\tau$ ms^{-1}) of K currents at E = 60, 80, 100, and 120 mV plotted as a function of the concentration (mM) of tetrapentyyammonium (TPeA) in the internal solution. Temperature 8°C, experiment F78.47 (see also Fig. 3). Values of τ are the time constants obtained by fitting a single exponential to the declining portion of each current record.

and the voltage dependence of a(E) dominates. Values of the rate coefficients a(E) ms^{-1} mM^{-1} and b(E) ms^{-1} obtained from several experiments are summarized in Table IV. In the Discussion we argue that a(E) almost certainly characterizes the rate of the binding reaction, whereas the physical basis of b(E) is more complex, its value being partially determines by channel gating kinetics.

Reversibility of Block

Block by the TAA ions up to TPeA was largely reversible. For 15 experiments, in each of which 5 to 9 *I-E* relations were determined, the maximum isochronal conductance decreased from 0.101 ± 0.023 s cm^{-2} (mean ± SD) in the first control run to 0.85 ± 0.025 in the last. A

TABLE IV

RATE COEFFICIENTS DETERMINED FROM THE LINEAR RELATION BETWEEN THE RECIPROCAL TIME CONSTANTS AND THE TETRAALKYL-AMMONIUM ION CONCENTRATIONS*

Ion	Exp. No.	Concentrations used	E	a	b	b/a
		(mM)		*($ms^{-1} - mM^{-1}$)*	*(ms^{-1})*	*(mM)*
TEA	S78.16	0.1, 0.2, 1.0	120	0.56	0.31	0.6
	S78.18	0.1, 0.3, 1.0	120	0.47	0.68	1.4
TPrA	F78.34	0.1, 0.3, 1.0	120	0.94	0.17	0.2
TBA	F78.43	0.05, 0.1, 0.2, 0.5	120	1.06	0.02	0.02
			100	0.92	0.04	0.04
			80	0.80	0.04	0.05
TPeA	F78.45	0.05, 0.1, 0.2	120	1.21	0.01	0.01
	F78.47	0.05, 0.1, 0.2	120	1.18	0.01	0.01
			100	1.03	0.02	0.02
			80	0.86	0.03	0.03
			60	0.74	0.03	0.04

*Eq. 1 of text.

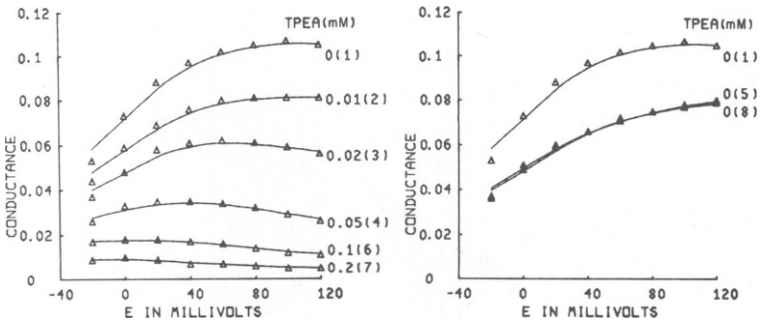

FIGURE 9 Conductance, g, as a function of transmembrane voltage, E, with various concentrations of tetrapentylammonium (TPeA) present internally. Concentrations of TPeA, in millimolar, are indicated on the figure. Order in which data were collected is shown in parentheses alongside each curve. Control runs are shown on a separate frame for clarity. Experiment F78.47, (see also Fig. 3). g was calculated from the step in current at the end of a 40-ms depolarizing pulse, when the voltage jumped back to the holding potential ($E = -80$ mV). Conductance is expressed in S/cm^2.

representative sequence of g-E relationships determined in a single experiment using TPeA is shown in Fig. 9. In two experiments with the THxA, even after thorough perfusion with the control solution ~50% of the initial control conductance had irreversibly vanished, so we attempted no quantitative experiments with this ion. Judging by the reduction in current from initial control values, THxA appeared to be somewhat more effective a blocker than TPeA.

Voltage-dependent Reduction in Isochronal Conductance

In Fig. 9, isochronal conductances, determined at the end of a 40-ms depolarizing voltage step, are plotted as a function of voltage, E, for an axon with various internal concentrations of TPeA. Control conductances, determined with K as the only internal cation, rise with increasing voltage until about $E = +60$ mV. At higher voltages, very little further increase in conductance was seen. With TPeA inside, conductance was depressed at all voltages examined, but there is a clear tendency for the reduction in conductance to be greater at higher voltages. Interpretation of the voltage dependence of the effect is complicated by the fact that the K conductance is not activated completely at all the voltages. (We are grateful to Dr. Paul Adams for drawing our attention to this point.) Further, 40 ms is too short a time to observe the steady state of the block at the lowest concentrations of TPeA employed. Both of these points are taken into account in the analysis of the voltage dependence of the steady-state block that follows.

An Analysis of the Voltage Dependence of the Steady-State Blocking Effect

For the higher concentrations used, the duration of the applied voltage pulses was sufficient to allow the blocking to reach a steady-state level. We analyzed the effect of voltage on the degree of block in the steady state by estimating the fraction, $f'/(f' + b)$, of channels that were activated by the applied coltage pulse and remained unblocked. This quantity was calculated from the experimentally measured conductances as indicated in the Appendix, Eq. A2. This estimate of the fraction of open channels that were not blocked is based on three assumptions: (*a*) that the normal voltage-dependent channel gating is unaffected by the

presence of the TAA ions, (*b*) that the control conductances at the highest voltages, $E = 100$ and 120 mV, give a reasonable approximation to the maximum conductance, and (*c*) that the single channel conductance is unchanged. With a single pair of free parameters for each ion species, one can then approximate the fraction of unblocked, open channels by the expression

$$f'/(f' + b) = 1/\{1 + \exp(sFE/RT)[X]/K_X\} \quad (4)$$

F, R, and T represent the Faraday, the ideal gas constant, and the absolute temperature respectively. [X] is the concentration of the blocking ion and K_X is the apparent dissociation constant for the blocking reaction at $E = 0$. The parameters s, the "apparent valence" of the blocking reaction, gives a measure of the voltage dependence of the block and may represent the fraction of the transmembrane voltage traversed by the univalent TAA ion as it effects the block.

Conductances used for this analysis were measured at the end of a voltage pulse whose duration, t, was long with respect to the time constant, τ, for the decay of the current. In the worst case (0.05 mM TPeA), $t/\tau \simeq 3$. With ~50% block at steady state, this implies that the value of $f'/(f' + b)$ determined at the end of the pulse should be within 0.03 of the

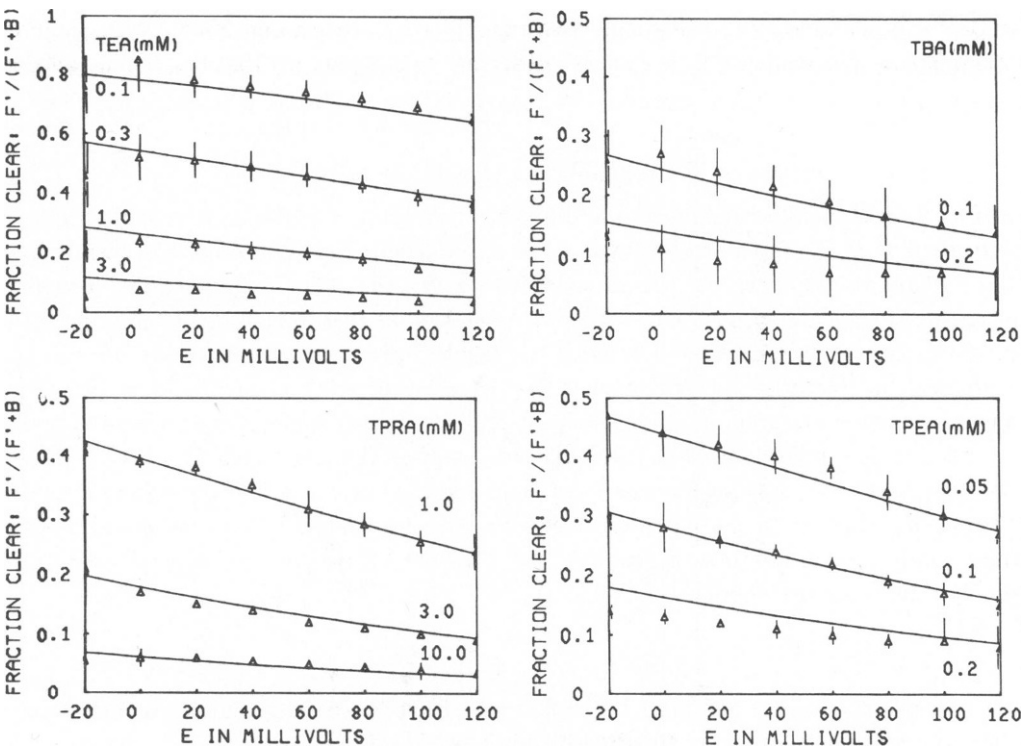

FIGURE 10 Estimated fraction of "open-gated" channels that are not blocked in the presence of tetraalkylammonium ions, as a function of voltage, at steady state. The "fraction clear" is calculated assuming that the normal, voltage-dependent channel gating is unaffected by the presence of the tetraalkylammonium ions (see text). Error bars indicate standard deviations where estimated and greater than symbol size. Number of axons used for each determination is given in Table V. The smooth curves are drawn using Eq. 4, with the parameter values shown in Table V.

steady-state value. In general, $t/\tau > 4$, implying a discrepancy of <0.01 between the end-of-pulse and steady-state values of $f'/(f' + b)$.

The lumped data, together with the best fit lines from the above equation, are shown in Fig. 10. The parameter values that gave the least squares fits appear in Table V. The parallel decrease in conductance with increasing voltage for all the ions is immediately obvious and is quantitatively expressed in the virtually identical values of s required to fit the data for different ions. Equally striking is the measure of the potency of the various ions provided by the apparent dissociation constants, K_X. The larger two members of the group block with much higher affinity than the smaller members of the group. The relationship between the apparent dissociation constants and ionic size are shown graphically Fig. 11. Silhouettes of Corey-Pauling-Koltun models, with alkyl side chains fully extended, indicate the maximum range of sizes among the group of TAA ions from TMA to TPeA.

DISCUSSION

General Properties of K Conductance Block by TAA Ions

There is no apparent qualitative difference between the mode of action of the various TAA ions present within the squid axon and that of the various related quaternary ammonium ions studied by other workers (Armstrong, 1969 and 1971; Armstrong and Hille, 1972; Shapiro, 1977). Briefly, the evidence from our own observations suggests (*a*) that the conductance is

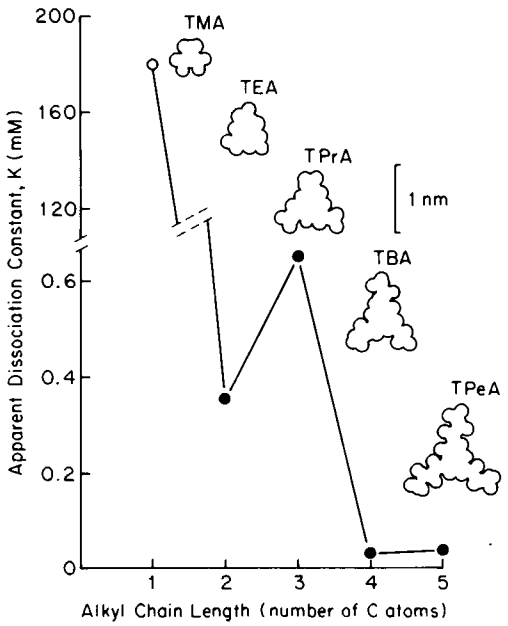

FIGURE 11 Apparent dissociation constants, at $E = 0$, as a function of alkyl chain length for the tetraalkylammonium ions. Values were obtained by fitting the data for steady-state block as shown in Fig. 10. The number of C atoms in each alkyl chain is n. Data for tetramethylammonium were added for comparison from work by French et al. (1979). Insets show silhouettes of Corey-Pauling-Koltun models of each ion to indicate relative sizes. Scale, alongside TPeA, is 10 Å.

susceptible to block by the TAA ions only after it is activated by depolarization of the membrane, (*b*) that the voltage-dependent turn-on of the conductance is unaffected by the presence of the TAA ions, (*c*) that normal turn-off of the conductance cannot occur with TAA ions bound to the blocking sites, and (*d*) that, from the concentration dependence of both the kinetics of block and the steady-state block, a single TAA ion interacts with each channel to block it. The lack of effect on the turn-on kinetics, and development of block after the conductance has been almost fully activated, is most easily illustrated for the larger ions that produce a slower, substantial block at low concentrations. Rapid block and unblock by TMA within a few microseconds of a voltage step suggest that the ions are able to bind to and dissociate from the blocking site without any change in the state of the activation "gates."

Comments on the Analysis

COMPLICATIONS DUE TO PERIAXONAL K ACCUMULATION The reversal potential, E_K, at the end of a pulse is a function both of the voltage E, and the blocker concentration due to variable K accumulation in the periaxonal space during the pulse (Frankenhaeuser and Hodgkin, 1956; Adelman et al., 1973). This results in a varying driving force, $(E - E_K)$, for the potassium current. Consequently, we used measurements of the conductance, rather than the current, to estimate the degree of steady-state block.

A further interpretive difficulty arises from the fact that external potassium ions probably modulate the block. Raising the K concentration on the *trans*-side of the membrane from the blocking ion does reduce the effect of a number of K conductance blockers (Armstrong and Binstock, 1965; Bezanilla and Armstrong, 1972; Hille, 1975; Adelman and French, 1978). In our experiments, even though the TAA-produced block increased with increasing voltage, the current never decreased. Thus, K accumulation increased with increasing voltage. This would tend to decrease the block at the higher voltages. Thus, it appears that there is genuine voltage dependence associated with the blocking reaction. Our estimate of s, reflecting the voltage dependence of the steady-state block if in error, is likely to be a little low.

EFFECT OF THE VOLTAGE DEPENDENCE OF GATING As we note in the Appendix, the apparent voltage dependence of a reaction in series with the voltage dependent channel opening could, in principle, result from the voltage dependence of the activation step. If we replace the left hand side of Eq. 4 by the relative conductance, g'/g (using the symbols defined in the Appendix) values of $s \simeq 0.2$–0.25 are required to fit the data. These agree closely with the value of $\delta(\equiv s) = 0.2$ used by Hille (1975) to fit the current-voltage data obtained from a frog node with TEA present internally. Similar values of $s \simeq 0.15$ (see Table V) required in Eq. 4 to fit the estimated fraction of the channels that is activated and not blocked affirms that (*a*) only a minor fraction of the variation in the relative conductance, over this range of voltage, can be attributed to the influence of voltage on the gating mechanism, and (*b*) the blocking reaction itself is voltage dependent.

KINETICS Arbitrarily fitting the exponential decline of the current or relative current in the presence of a TAA ion gives us a rigorous way of selecting conditions for analysis of the steady-state block by quantifying the rate of approach to the steady state. It does not, however, tell us what or how many physical processes influence the rate. At any particular voltage, the slope a in Eq. 1 represents exactly that contribution to the rate, $1/\tau$, that is proportional to TAA concentration. The intercept, b, on the other hand, incorporates

TABLE V
PARAMETER VALUES FOR LEAST-SQUARES FIT OF THE DATA TO EQ. 4 OF THE TEXT*

Ion	Concentrations used for fit (No. of exps. in parentheses)	S	K_x
			(mM)
TMA‡	100	0.3–0.4	~100
TEA	0.1(4), 0.3(3), 1.0(3), 3.0(3), 10.0(4)	0.14	0.36
TPrA	1.0(2), 3.0(1), 10.0(2)	0.15	0.65
TBA	0.1(4), 0.2(2)	0.16	0.032
TPeA	0.05(3), 0.1(3), 0.2(3)	0.15	0.039

*Reaction scheme is indicated in the Appendix.
‡From instantaneous *I–E* data (French et al., 1979).

all contributions to $1/\tau$ that are independent of TAA concentration and may well be affected by more than one process. While the linear relationships between $1/\tau$ and the [TAA] shown in Fig. 8 tempt one to think of a rate-limiting binding reaction between one TAA ion and a channel-blocking site, there are several observations that caution against this simple interpretation of the rate coefficients in Table IV.

(*a*) At concentrations between 1 and 10 mM, $1/\tau$ for TEA and TPrA did not continue to go up linearly with concentration but began to level off. In the context of the reaction scheme given in the Appendix, this suggests that the concentration independent channel opening is becoming the limiting step.

(*b*) In the cases of TBA and TPeA, for which the temporal separation between the activation and blocking processes is greatest, $1/\tau$ was obtained at several different concentrations and voltages. For a barrier symmetric with respect to the applied electric field, the blocking rate coefficients may empirically be written in the form $a(E) = a_0 \exp(sFE/2RT)$, where a_0 is the rate at $E = 0$, and $s \sim 0.3$–0.4. Comparison of these s values with those obtained by fitting steady-state data suggests that the activation energy barrier (or barriers) determining these rates cannot be those which determine the binding and dissociation rates that make up the apparent dissociation constants determined from the steady-state binding. The larger influence of E on the rate coefficients suggests that the highly voltage-dependent channel gating is partially limiting. In general, the voltage dependence of the transition from the closed to the open state will contribute some voltage dependence to the rate of decline of the currents regardless of whether or not the blocking reaction is itself voltage dependent (cf. Goldman, 1976; Bezanilla and Armstrong, 1977; Armstrong and Bezanilla, 1977). This point is illustrated by the simulation shown in Fig. 12.

(*c*) If the rates a and b represent only the binding step, the ratio b/a would give the dissociation constant at the given voltage. The sequence of values of b/a at $E = 120$ (Table IV) is close to, but not identical with, the sequence of apparent dissociation constants determined from the steady-state block. In fact, the b/a values decrease monotonically with increasing size of the TAA ions. That these values do reflect, as nearly as they do, the K_x values of Table V, argues that the rate coefficients a and b do primarily, but not solely, reflect the rates of the TAA binding and dissociation.

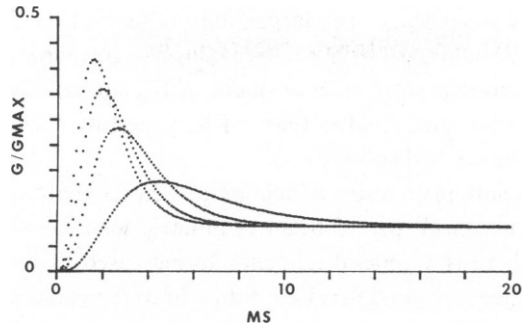

FIGURE 12 Time-course of conductance, expressed as a fraction of the maximum value, calculated assuming the channels open with Hodgkin-Huxley (1952) n^4 kinetics and are then blocked by a voltage-independent blocking agent. Note the voltage-dependent rate of decay of the conductance. This is due to the voltage-dependent turn-on of the conductance. Conditions assumed: holding potential, $E = -80$ mV; voltage steps to $E = 0$, 40, 80, and 120 mV; binding rate for block, 1.0 ms^{-1}; dissociation rate, 0.1 ms^{-1}.

Site of Action and Potency of Block

The notion that the TAA ions reduce the potassium conductance by entering and occluding the path of ion permeation after the channel "gates" have opened is consistent with our observations. Indeed, it seems to be the simplest and most likely physical explanation. One observation that has been used to support this view is that a high external K concentration reduced the effectiveness of quaternary ammonium blockers. This is reflected in the relatively low value that we obtained for the apparent dissociation constant for TEA block ($K_{TEA} \sim 0.4$ mM at $E = 0$). For comparison, one can estimate a value from the data of Armstrong (1966, Fig. 7), assuming that the rate constants strictly represent the binding and unbinding of TEA. In those experiments, with 440 mM K externally, $K_{TEA} \sim 5$–6 mM at $E = 80$–100 mV. In Fig. 2 of the same paper one sees that 0.6–1.2 mM TEA was required to reduce steady-state currents to ~50% of the control value for an axon bathed in 10 mM K artificial seawater. This implies a K_{TEA} very close to that which we obtained. Hille (1975) used a value of $K_{TEA} = 5$ mM (at $E = 0$) to fit *I-E* data from frog myelinated nerve with internodes cut in TEA-containing solutions. We are not sure whether this value represents a genuine difference in affinity between the inner TEA binding site in squid and frog, or arises from the different experimental and analytic approaches.

The voltage dependence ($s \sim 0.15$, see Table V) of K channel block by all of the TAA ions, with 2–5 carbon *n*-alkyl side chains, is virtually identical. Together with the qualitative similarities between their kinetics of action, this argues strongly that they act at the same site. The specific physical basis of the voltage dependence is not clear. Three possibilities are: (*a*) that a TAA ion simply moves through some fraction of the applied voltage drop to reach its binding site, (*b*) that the TAA ion binds outside the applied field and, when it does so, causes an obligatory movement of a K ion into the field, (a strict "knock on" mechanism), or (*c*) that some charged or polar entity must move in the field to make the blocking site available for TAA binding. Little can be said for or against the third possibility. As already noted, external K ions can modulate QA block, suggesting that the second possibility is at least partly true. Finally, the observation that TMA, the smallest of the TAA ions, shows a voltage dependence

of block about twice as steep as for the larger ions suggests that the first possibility may contribute. French and Shoukimas (1979) noted an inverse correlation between ionic size and voltage dependence of action among an assortment of K channel blockers. This is consistent with the simple notion that ions, smaller than TEA, penetrate further and hence are more strongly influenced by the applied voltage.

The most dramatic qualitative result which we present is that, at least up to the size of THxA, there is no exclusion of any of these symmetric ions from the internal K channel blocking site. In fact, there is a general tendency to increased potency with increasing size. TMA is ~100-fold less potent than TEA. Our data add to the evidence favoring Armstrong's (1969) suggestion, based on experiments with ions of the form $(C_2H_5)N^+$—R, that hydrophobic interactions with the membrane are crucial in conferring on these blocking ions the ability to bind strongly. Also in agreement with Armstrong's work, our kinetic analysis suggests that blocking potency depends more on binding strength than on ease of access to the blocking site. Estimates of the dissociation rate drop dramatically as ion size increases (Table IV), whereas all the calculated rate coefficients for the binding are within a factor of ~2 of each other. The external TEA receptor in frog nodal membrane shows quite different selectivity, interacting only weakly with TPrA and TBA (Hille, 1967).

Based on the steady-state block, our analysis indicates that the dependence of binding potency on size is not perfectly monotonic (K_{TEA} is less K_{TPrA}). At this point we hesitate to attribute a great deal of significance to this difference for the following reasons: (*a*) the variation in apparent dissociation constants over the whole TAA series (>2,000 ×) is much larger than the change from TEA to TPrA (<2 ×), (*b*) the sequence of values of b/a at $E = +120$ mV is monotonic (see Table IV). If the difference between K_{TEA} and K_{TPrA} is confirmed by more extensive experiments, it would suggest that a diameter of 8 Å does significantly favor entry into, and binding with, the inner mouth of the K channel in accordance with Armstrong's (1975 *a* and *b*) suggestion.

Though we have no direct experimental measurements, it is of interest to ask whether TMA and similar "instantaneous" blocking ions (Bezanilla and Armstrong, 1972; French and Wells, 1977; French et al. 1979) are really faster blockers. The circumstantial evidence from the behavior of the larger TAA ions and other quarternary ammonium ions (Armstrong, 1969) suggests that the "instantaneous" blocking ions are simply faster at dissociating from the channels, making them less potent. At the high concentrations necessary to observe substantial block, then, the binding reaction is forced to proceed very rapidly. The rate constant for binding may show very little variation from one ion to another.

Implications for K channel Structure

If there is a rigid and dense molecular structure forming an inner mouth for the K channel, it would have to be at least 50% larger than the lower limit of 8Å implied by Armstrong (1975 *a* and *b*). Since the alkyl chains of the larger TAA ions are quite flexible, with the possibility of free rotation around the C—C bonds, we do not know the shape that ions adopt when interacting with the membrane. However, even if the alkyl chains of a TPeA molecule were folded, without distortion of the bond angles, to assume a minimum diameter, the diameter would be ~12 Å. The following simple calculation suggests that the channel cannot have an aqueous lumen with diameter large enough to accomodate these ions.

We assume, for the moment, that the sole cause of the voltage dependence of block is the movement of the TAA ion into the field. If this is so, then the channel "mouth" accessible to TAA ions must account for 15% of the resistance of a single channel. Taking 10^{11} Ω as an approximation to the channel resistance, r (Neher and Stevens, 1977), we calculate the length, L, of the channel mouth. We further assume that lumen's cross-section is circular, and that it is filled with an electrolyte of resistivity $\rho = 20$ Ω cm (approximately that of seawater). Under these assumptions $L = 0.15\, r\pi\, (D/2)^2/\rho \sim 850$ Å, where D is taken to be 12 Å. Even for $D = 8$ Å, the size necessary to accomodate TEA, $L \sim 380$ Å! Both numbers are impossibly large. While one does not expect macroscopic solution properties to provide a precise description for a pore of molecular dimensions, experimental evidence suggests that the calculated values should not be far from the truth. For the gramicidin channel, with a diameter of only 4 Å, macroscopic viscosity leads to a prediction of water permeability within a factor of 4 of the experimentally measured value (Finkelstein and Rosenberg, 1979). Using observed values of the unit conductances of alamethicin, Eisenberg et al. (1977) calculated apparent radii for the various conducting states, based on macroscopic solution conductance, that were within a factor of ~2 of the radii of the largest permeant ions for each state.

Even though a large lumen is unlikely, it is still possible that the voltage dependence of block is due to the movement of TAA ions into the channels. An ion may be able to enter even though the aqueous channel lumen is too narrow to encompass the whole ion with its alkyl side chains. A structure composed of several dynamically interacting subunits embedded in lipid might accomodate ions of a variety of sizes and shapes and allow the hydrophobic parts of the blocking ion to interact with the membrane lipid. Heyer et al. (1976, Fig. 15) envision a similar possibility to explain how QA ions might pass through and inactivate a monazomycin channel. We imagine the TAA ions simply entering and occluding the K channel rather than passing through. The "fantasy" of Heyer et al. (1976) could easily be extended to visualize two or more alkyl chains of a TAA ion sliding between channel subunits while the central N atom actually penetrated a short distance into the lumen of the channel.

Our results clearly prompt us to ask the experimental question as to whether or not large symmetric ions with hydrophilic surface groups can block the K conductance. The implication of our work is that the K channel could not accommodate such an ion of the size of say, TPeA. Our work also underlies the mystery raised by Shapiro (1977): How is it that an ion of the size of *N*-methylstrychnine can block with such steep dependence on applied voltage? Because of the relatively slight influence of voltage on the action of the TAA ions we presume our results to reflect only properties of the innermost part of the K channel, and not those of the outer part of the channel which appears to be responsible for the channel's extreme selectivity for permeant ions (Armstrong, 1975 *a* and *b*).

APPENDIX

From our own observations, and following the lead of Armstrong (1966 and 1969), we suppose that the normal voltage-dependent activation of the K conductance occurs unchanged in the presence of the TAA ions. Thus, with K^+ as the only internal cation, we have

$$\begin{array}{ccc} \text{Closed} & = & \text{Open} \\ 1 - f & & f \end{array}$$

and in the presence of one of the TAA ions,

$$\begin{array}{ccccc} \text{Closed} & \rightleftharpoons & \text{Open} + \text{TAA} & \rightleftharpoons & \text{Blocked} \\ 1 - (f' + b) & & f' & & b \end{array}$$

The symbols below the equations represent the fraction of the channels in each of the states, at steady state, for any given voltage E. We presume conductance is proportional to the number of open channels, and that the single channel conductance does not change. Thus, if g and g' represent the control conductance and the conductance in the presence of one of TAA species, we have

$$f = g/\bar{g} \text{ and } f' = g'/\bar{g},$$

where $\bar{g}$ is the conductance when all the channels are activated.

In the steady state, at any given voltage, using the assumption that the TAA ions do not affect the voltage dependence of gating:

$$f/(1 - f) = f'/[1 - (f' + b)]. \tag{A1}$$

Rearranging,

$$f'/(f' + b) = f'/[1 - (f'/f)(1 - f)]. \tag{A2}$$

All quantities on the right hand side of eq. A2 are experimentally measurable, so we have the desired expression for the fraction of open-gated channels that are not blocked. Note that if $f = 1$, then $f'/(f' + b) = f' = g'/g$. Thus, it is only when all channels are activated at the voltages of interest that the relative conductance, g'/g, would accurately reflect the voltage dependence of the transition from the open state to the blocked state (see also the discussion of the voltage dependence of sodium channel inactivation by Bezanilla and Armstrong, 1977, and Armstrong and Bezanilla, 1977).

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