Time Course of TEA^+-Induced Anomalous Rectification in Squid Giant Axons

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ABSTRACT Changes in the voltage clamp currents of squid giant axons wrought by low axoplasmic TEA^+ (tetraethylammonium chloride) concentrations (0.3 mM and above) are described. They are: (*a*) For positive steps from the resting potential in sea water, the K^+ current increases, decreases, then increases, instead of increasing monotonically. (*b*) For positive steps from the resting potential in 440 mM external K^+, the current has an exponentially decaying component, whose decay rate increases with axoplasmic [TEA^+]. The control currents increase monotonically. (*c*) For negative steps from the resting potential in 440 mM external K^+, the current record has a peak followed by a decay that is slow relative to the control. The control record decreases monotonically. Qualitatively these findings can be described by a simple kinetic model, from which, with one assumption, it is possible to calculate the rate at which K^+ ions move through the K^+ channels. An interesting conclusion from (*c*) is that the channels cannot be closed by the normal voltage-sensitive mechanism (described by Hodgkin and Huxley) until they are free of TEA^+.

INTRODUCTION

In a previous paper (Armstrong and Binstock, 1965) it was demonstrated that tetraethylammonium chloride (TEA^+) injected into axons of the squid *Loligo pealii* to an axoplasmic concentration of approximately 40 mM prevented the flow of outward K^+ current. It was proposed that TEA^+ ions tend to enter the membrane from the axoplasm and stop up the K^+ channels unless the channels are swept clear of TEA^+ by inrushing K^+ ions. At an axoplasmic TEA^+ concentration of 40 mM the stopping up process occurred too fast to be observed; i.e., following a positive step of membrane potential (i.e., inside made more positive), TEA^+ entered and stopped up any initially TEA^+-free channels so rapidly that no outward K^+ current was ever seen. The experiments reported here were prompted by the hope that at lower axoplasmic TEA^+ concentrations, the K^+ channels might be stopped up more slowly, and

that the rate of entry of TEA^+ ions into the channels could be deduced from the rate of decline of the K^+ current.

METHODS

Squid of species *Dosidicus gigas* were caught a mile off the coast of Chile by fishermen trained to remove a large section of the mantle containing the giant axons. This section was placed in ice cold sea water, and dissection was begun 4 to 6 hr later. Numerous giant axons radiate from the stellate ganglion, and of these the most suitable (though not always the largest) was the one nearest the midline. Average diameter of this axon from December through February was about 0.8 mm, and in approximately one squid out of five its diameter was 1 mm or more (largest, 1.2 mm). Large diameter and absence of branches for more than 10 cm made dissection quite easy. Survival for 8 hr under experimental conditions was not unusual.

Voltage clamp apparatus and chamber were similar to that described previously (Cole and Moore, 1960; Armstrong and Binstock, 1964), except for modifications of the chamber to accommodate the larger axons. Modifications included a trough for the axon of larger width (5 mm) and length (50 mm), and slightly longer current-measuring (5 mm) and guard (6 mm) electrodes. The terminal 3 mm on either end of the trough was an air gap, to prevent shunting of the membrane potential by the damaged ends. This made it possible to work with a shorter segment of axon, but had the disadvantage of putting a high resistance in series with a portion of the membrane capacity. The measuring region was shielded from the ends by the guard electrodes, however, and the gaps had no effect on the voltage clamp currents, as was ascertained by filling the gaps with sea water from time to time. Filling the gaps decreased action potential magnitude by as much as 10 mv.

Injection of TEA^+ (tetraethylammonium chloride, Eastman Kodak) was accomplished by pushing a cannula from one end of the axon segment to the other, and withdrawing it in steps while TEA^+ (in water) was injected from a microsyringe. Total injected volume was usually 2 μl, into (on the average) an axoplasmic volume of 35 μl. With care this could be done a number of times. The axons were often large enough to contain the cannula and the voltage clamp electrode side by side, but with smaller axons it was necessary to withdraw the electrode each time.

All experiments were performed at 6 to 10°C. External solutions had the composition previously noted (Armstrong and Binstock, 1965) except for the Tris concentration, which was 5 mM. No corrections were made for liquid junction potentials. Action potentials were measured under space clamp conditions.

RESULTS

Resting and Action Potentials Initial and final values of the resting potential in artificial sea water (RP_{ASW}) and action potential are given in Table I for most of the fibers tested. (Potential is that of the inside of the membrane with outside zero.) RP_{ASW} in the early experiments (December and early January) was 5 to 10 mv more negative than in the later experiments (January and February) when most of the fibers had a resting potential near −45

mv. Resting potentials of −40 to −45 mv were also reported by Rojas and Luxoro (1963). Resting potential in 440 mM external K^+ (RP_{440K}, Table I) tended to be more positive in the later experiments, again by 10 mv or more. This suggests that in the later experiments the internal $[K^+]$ was lower by a factor of approximately 0.7. Unfortunately, no chemical analyses were made.

Fig. 1 illustrates the normal action potential and its alteration following TEA^+ injection, which has the same effect as in *Loligo pealii* (Tasaki and

TABLE I

Experiment	AP	RP_{ASW}	RP_{440K}
2	108 to 70	−58 to −50	
3	108 to 70	−59 to −51	
4	110	−57	
5	110 to 88	−57 to −47	
7	110 to 86	−54 to −57	
8	108 to 60	−52 to −44	6
9	110 to 90	−44 to −43	16
10	110 to 80	−46 to −40	13
13	92 to 70*	−56 to −48*	0
14	100 to 0*	−56 to −36*	0
15	—	−48 to −26‡	3
16	110 to 0	−55 to −20	3
18	100 to 0	−53 to −42	5
19	112 to 0	−53 to −34	12
25	104 to 70	−47 to −37	12
26	104 to 60	−48 to −44	14
28	100 to 60	−48 to −36	9
31	100 to 0	−45 to −32	14
32	100 to 0	−42 to −25	17
33	104 to 84	−38 to −33	
34	100	−41	16
35	110 to 88	−46 to −33	16
36	110 to 100	−41 to −39	

* Initial determination followed TEA^+ injection.
‡ Initial determination followed exposure to 440 mM K.

Hagiwara, 1957). TEA^+ injection had little if any effect on either RP_{ASW} or RP_{440K}.

Voltage Clamp Experiments The current patterns of normal *Dosidicus gigas* axons (Fig. 2, 0 TEA^+) following positive steps of membrane potential (V_m) closely resemble those of axons from *Loligo pealii* and *Loligo forbesi* (e.g. Cole and Moore, 1960; Hodgkin and Huxley, 1952 *a*). Replacement of the external Na^+ with $choline^+$ indicates that the initial current is carried, as in the other species, by Na^+ (Hodgkin and Huxley, 1952 *a*), and it will be assumed that the later current is carried by K^+ (Hodgkin and Huxley, 1952 *a*).

TEA^+ at increasing axoplasmic concentrations affects chiefly the K^+

current (Fig. 2), which is decreased in magnitude, and for axoplasmic [TEA^+] between 0.6 and 2.4 mM (or higher in other experiments) has an unusual hump 2 to 1.5 msec after the step; i.e., the K^+ current increases, decreases, then increases slowly. A possible interpretation of this behavior (see Discussion) is that as the K^+ channels open in response to depolarization (Hodgkin and Huxley, 1952 *b*), some of them are blocked by TEA^+, and the slope of the current-time curve at any instant depends on the relative rates of opening and stopping up. With 27 mM TEA^+ (not shown) there is no discernible K^+ current (cf. Armstrong and Binstock, 1965). Usually there was little change in the Na^+ current, except for a decrease in E_{Na} which is probably not directly related to TEA^+ injection.

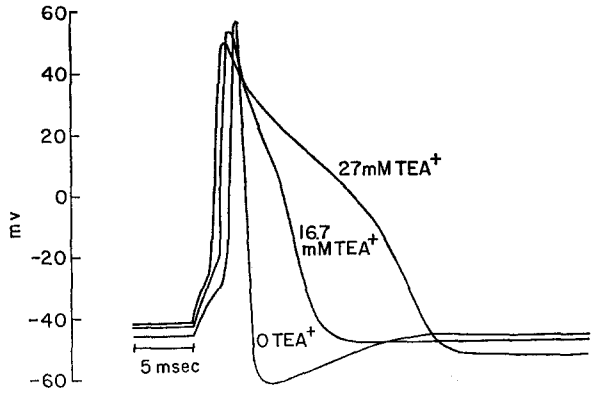

FIGURE 1. Action potentials recorded under space clamp conditions, with axoplasmic [TEA^+] as given. Axon 9. Temperature 7 to 8°C.

The stopping up of the K^+ channels by TEA^+ (if this is indeed the case) is easier to see in the currents that follow positive steps of potential applied to a fiber resting in 440 mM K^+. Such records are simpler for two reasons: the normal opening and closing mechanism of most of the channels is open (because the fiber is depolarized), and remains so during a positive step from RP_{440K}; and there is no Na^+ current. An experiment of this kind is shown in Fig. 3. The fiber was maintained at RP_{440K} until the application of the step, when V_m was abruptly changed to the voltage given in the figure. In the control curves for positive steps (Fig. 3 *a*), there is a rapid downsweep of the trace as the capacity current decays (completed about 200 μsec after the step), and the current magnitude then increases slightly. After TEA^+ injection, the current continues to decrease after the capacity transient, and only after a half millisecond or so begins to increase very slowly. The current decline (after the capacity transient) gives an indication of the rate at which TEA^+ is entering the channels (see Discussion).

Fig. 3 also shows the (inward) currents that follow negative steps of potential applied to the same fiber, resting in 440 mM K^+. In the control curve (Fig. 3 *a*) there is a brief downstroke of the trace (increase in current magnitude, possibly the result of an oscillation in the feedback system), and the current

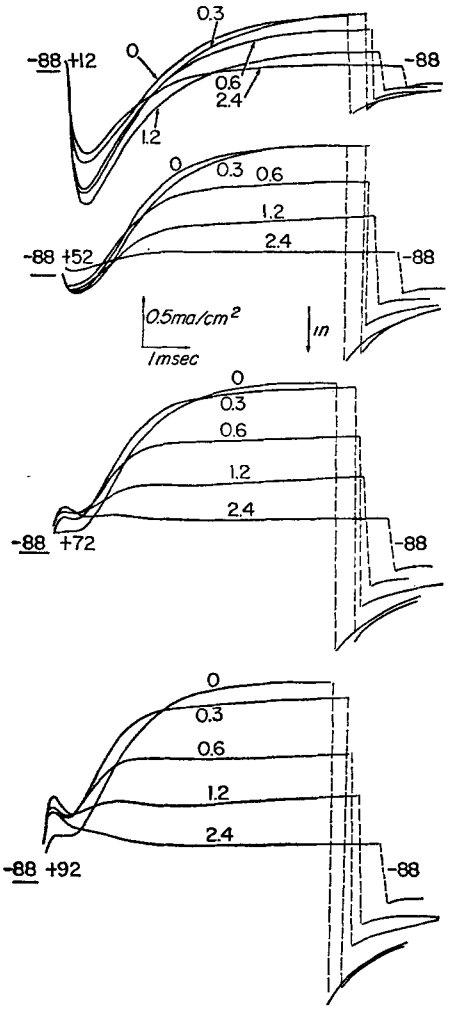

FIGURE 2. Voltage clamp currents of axon 26 in ASW, at several axoplasmic TEA$^+$ concentrations (numerals give concentration in mM). Positive step (to potential given) was preceded by a conditioning step to −88 mv. Temperature 7°C.

then declines, rapidly at first, and then more slowly. This is similar to the behavior of axons from *Loligo*, except that the initial component of current decline is much faster with *Dosidicus* axons. After TEA$^+$ injection (1.2 mM, Fig. 3 *b*, inward currents), the current continues to increase in magnitude after the initial downstroke of the trace, leading to a peak about 0.5 msec after the step. This increase of the current until 0.5 msec probably means

that some of the K^+ channels are being swept clear of TEA^+ ions by an influx of K^+ ions (see Discussion). After the peak at 0.5 msec, the current declines, but considerably more slowly than in the controls.

The current curves of Fig. 4 are similar to those just described except that two voltage steps were applied to the fiber, as indicated by the numbers in

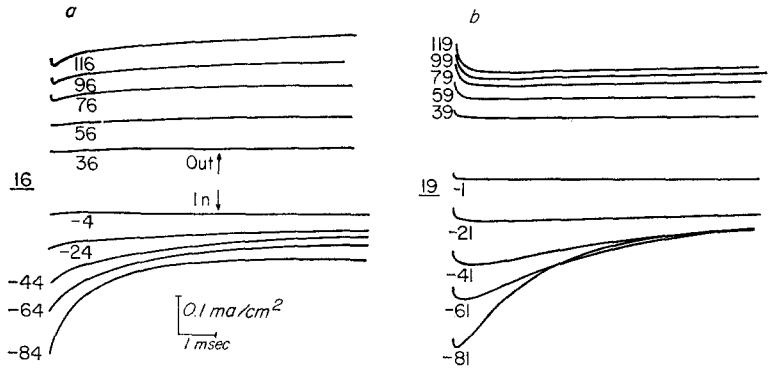

FIGURE 3. Voltage clamp currents of axon 35 in 440 mM external K^+. (*a*) no TEA^+, (*b*) 1.2 mM axoplasmic TEA^+. Fiber was held at RP_{440K} until the application of the step to the potential given. Temperature 6°C.

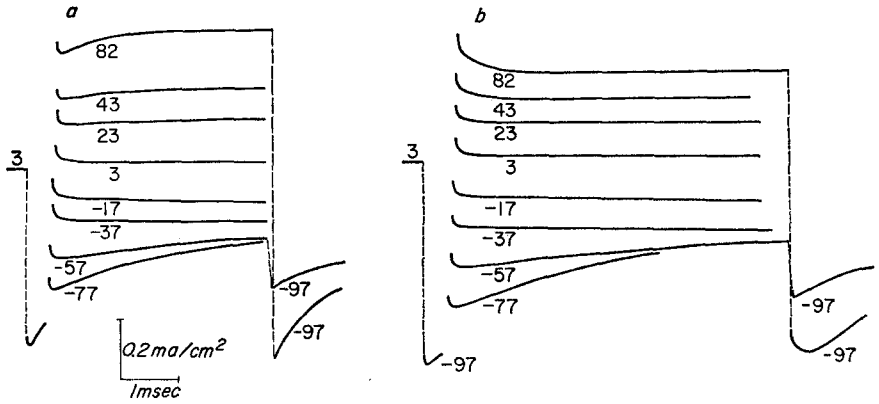

FIGURE 4. Voltage clamp currents of axon 16 in 440 mM external K^+. (*a*) no TEA^+, (*b*) 0.54 mM axoplasmic [TEA^+]. Test step to potential given was preceded by a brief conditioning step to −97 mv, as indicated in the figure. Potential was returned to −97 mv after the test step. Temperature 8°C.

the figure: a brief conditioning step to −97 mv, followed by a second step to the potential given (the test step). In Fig. 4 *b* it will be noted that there is no peak in the current record following the conditioning step to −97 mv. However, if the test step was positive and sufficiently large, there was a prominent peak when, after the test step, the potential was returned to −97 mv. This is to be expected if the number of TEA^+-occluded channels increases when

$V_m - E_K$ is made more positive, and if the peak, on returning to −97 mv, results from TEA^+ ions being swept out of the channels. The current tails for positive test steps were somewhat larger in amplitude when the double pulse procedure was used.

Current curves in 440 mM K^+ at several different axoplasmic TEA^+ concentrations are shown in Fig. 5. The outward currents are records of the double pulse experiment, for the axon of Fig. 2; i.e., the positive step to 114 mv was preceded by a brief step to −86 mv. As $[TEA^+]$ increases, the rate of decline of the current tails following positive steps is progressively faster, and

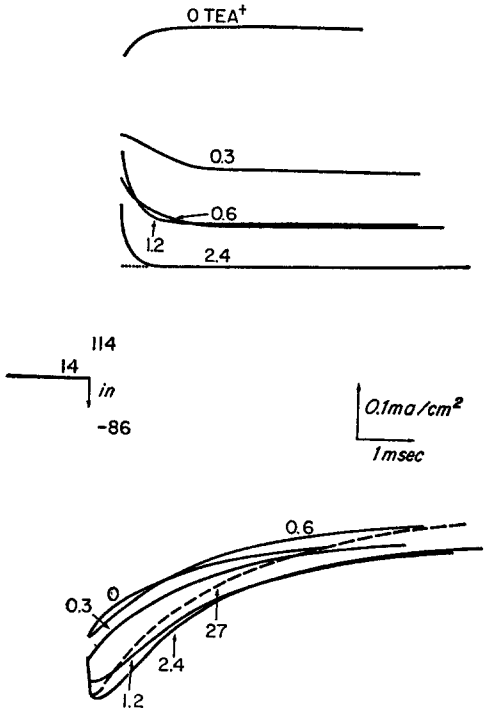

FIGURE 5. Voltage clamp currents of axon 26 in 440 mM external K^+, at axoplasmic $[TEA^+]$ given (in mM). Outward and inward currents are respectively for steps of 100 and −100 mv from RP_{440K}. Positive steps were preceded by a step of approximately 0.5 msec to −86 mv. Temperature 7°C. Dashed line labeled 27 (mM TEA^+), axon 9, 8°C. Dotted line, see text.

the minimum value of the current generally smaller. For concentrations 0.6 through 2.4 mM, each of these curves could be fitted by a decaying exponential and a line with a slight positive slope (e.g., for the 2.4 mM TEA^+ curve, the dotted line in Fig. 5). Semilog plots of the excess of the current over these lines are given in Fig. 6. Only the 0.3 mM curve is not exponential (i.e. not a straight line in the semilog plot). Time constants for four such experiments are given in Table II, for positive steps of 79 and 100 mv and several TEA^+ concentrations. In all cases save one the time constant decreases with increasing $[TEA^+]$.

For negative potential steps (Fig. 5), a peak appears in the current curve

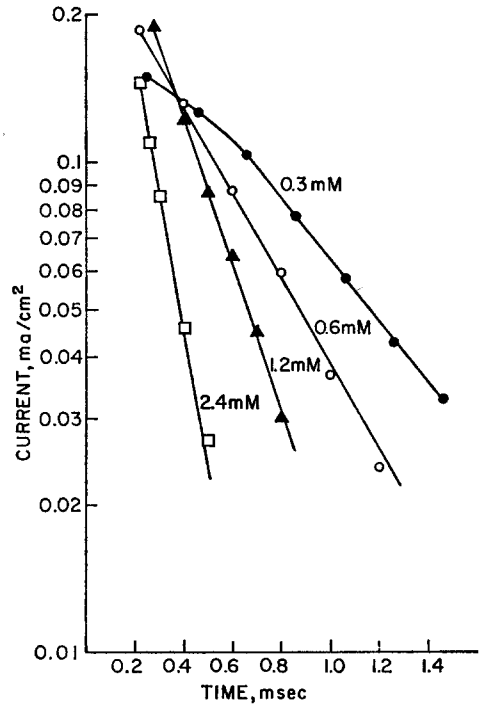

FIGURE 6. Semilogarithmic plots of the exponential portion of the current records for positive steps from Fig. 5. Axon 26, 7°C.

at an axoplasmic [TEA^+] of 1.2 mM, and the subsequent current decline becomes slower. Increasing [TEA^+] beyond 2.4 mM did not further slow the current decay, as can be seen by comparing the dashed curve (27 mM [TEA^+]) with the 2.4 mM curve.

TABLE II

Experiment	[TEA^+]	τ at RP_{440K} + 79 mv	τ at RP_{440K} + 100 mv	Temperature
	mM	*msec*	*msec*	°C
15	0.54	0.39	0.43	7
	1.08	0.30	0.28	8
	1.62	0.23	0.30	8
16	0.54	0.42	0.44	8
	1.08	0.30	0.28	8
26	0.6	0.51	0.51	7
	1.2	0.31	0.31	7
	2.4	0.16	0.16	7
35	0.6	0.52	0.42	6
	1.2	0.32	0.30	6

DISCUSSION

It was demonstrated previously (Armstrong and Binstock, 1965) that when TEA^+ is present inside an axon, the K^+ channels have two "gates" instead of the normal one, and that both must be open if the channel is to conduct current. One of the gates is the normal opening and closing mechanism, described by Hodgkin and Huxley (1952 *b*). In their description the probability that this gate is open is given by n^4, and for want of a better term, this will be called the n^4 gate. The other gate, the TEA^+ gate, functions as a diode in series with the n^4 gate: open to inward K^+ current and closed to outward K^+ current. It was considered (Armstrong and Binstock, 1965) that this gate opened or closed instantaneously in response to a change in the direction of the current, but it is clear from the evidence presented here that this is not always the case. It was noted in passing that the two gates are not entirely independent, for TEA^+ injection affects the kinetics of the n^4 gate. The data presented here help to clarify the relation between the two gates, and suggest a kinetic model with four possible states for the K^+ channels.

The four states are:

1. *XC*: The n^4 gate is closed and no ions may pass into or through the channel.

2. K*C*: Both the n^4 gate and the TEA^+ gate are open, and K^+ ions may pass through, or TEA^+ ions into, the channel.

3. TEA*C*: The n^4 gate is open, but the TEA^+ gate is closed. A closed TEA^+ gate means that a TEA^+ ion has entered the channel from the axoplasm and occluded it. If, for example, the channel is a long pore (Hodgkin and Keynes, 1955), TEA^+ might occupy the inner one or two sites of the pore and be unable to proceed further in the outward direction.

4. The channel is "inactivated" by an unknown mechanism, and ions cannot pass through.

The relation between these four states can be represented by an arrow diagram:

$$XC \rightleftarrows KC \underset{l}{\overset{k}{\rightleftarrows}} TEAC$$
$$\Updownarrow$$
inactivated

In this scheme, *XC* and *KC* are respectively the closed and open positions of the n^4 gate, and the kinetics of the transition from one to the other are given by Hodgkin and Huxley (1952 *b*). As will be discussed in (*c*) below, the K*C* $\rightleftarrows$ TEA*C* transition is a first-order kinetic process, and can be described by two rate constants, k and l. k is the rate constant of the occlusion of the

channels by TEA^+, and depends on the rate at which ions enter the channel from the axoplasm (and therefore on $V_m - E_K$) and on the fraction of these ions which are TEA^+; i.e. on the TEA^+ concentration. Dependence of the TEA^+ entry rate on $V_m - E_K$ seems unreasonable at first, but is understandable. If influx is very large compared to efflux, the channel will be completely filled with ions from outside, and none of these will be TEA^+. If efflux is comparable to or larger than influx, TEA^+ ions will enter the channel, since a fraction of the ions entering from the inside will be TEA^+. k increases as V_m is made more positive, and the rate at which the channels are stopped up by TEA^+ is given by the product $k \cdot KC$. Ions can move neither in nor out through a TEA^+-occluded channel until the TEA^+ ion is dislodged by a K^+ ion moving inward. l, the rate constant for the removal of TEA^+ from the channels, thus depends on the pressure which drives K^+ ions inward; i.e., on $V_m - E_K$. l increases as V_m is made more negative, and the rate at which TEA^+ leaves the channels is given by $l \cdot TEAC$.

The inactivated state (Grundfest, 1960) is necessary to account for the relatively low value of g_K when the fiber is in 440 mM K. The rate constants governing transition $KC \rightleftarrows$ inactivated are small (Ehrenstein and Gilbert, 1966) and it will be assumed that for present purposes they are insignificantly small.

Many of the qualitative predictions of this scheme can be observed in the data. (*a*) When the membrane is at RP_{ASW}, most of the channels are in state XC. Depolarization opens the n^4 gate of some of the channels, and if TEA^+ is present, some of the KC channels will become $TEAC$ (i.e. they will be stopped up by TEA^+), at a rate given by the product $k \cdot KC$. In Fig. 2, for example, for the depolarization to 91 mv at TEA^+ concentrations 0.6 through 2.4 mM, the hump in the K^+ current (about 2 to 1.5 msec after the step) can be explained by saying that rate $XC \rightarrow KC$ is initially large compared to rate $KC \rightarrow TEAC$, making dKC/dt (and dI/dt) positive. As KC increases, rate $KC \rightarrow TEAC$ ($= k \cdot KC$) increases, and at 2 to 1.5 msec becomes larger than $XC \rightarrow KC$, making dKC/dt and dI/dt negative. Increasing $[TEA^+]$ from 0.6 to 2.4 mM makes k larger, causes the hump to occur progressively earlier, and progressively decreases the number of channels open at long times after the step.

(*b*) In 440 mM K^+ the membrane is depolarized, making n^4 approximately unity. Therefore few channels are XC, while many are KC. Following a positive step from RP_{440K}, rate $XC \rightarrow KC$ is therefore smaller, except at very low $[TEA^+]$, than rate $KC \rightarrow TEAC$, because of the low value of XC. dKC/dt is therefore negative, until $KC \rightarrow TEAC$ is equaled by $TEAC \rightarrow KC$ (with a small contribution from $XC \rightarrow KC$).

(*c*) Following a positive step from RP_{440K}, the current curve approaches its infinity value (actually a line—see Results) exponentially for $[TEA^+]$ be-

tween 0.6 and 2.4 mM (and, presumably, at higher concentrations). The scheme proposed will reproduce this behavior if rate $XC \rightarrow KC$ is very small compared to $KC \rightarrow TEAC$, and if $KC \rightleftarrows TEAC$ obeys first-order kinetics. The scheme also reproduces the observed decrease of the "infinity value" of the current as [TEA$^+$] increases.

(*d*) Following a negative step from RP_{440K}, rate $KC \rightarrow XC$ is large while rate $TEAC \rightarrow KC$ ($= l \cdot TEAC$) depends on TEA*C*, and thus on [TEA$^+$]. At low [TEA$^+$] (below approximately 1.2 mM) rate $KC \rightarrow XC$ is always the larger, and the current decreases monotonically. Above this [TEA$^+$], rate $TEAC \rightarrow KC$ is initially the larger, making dKC/dt initially positive. In this case, as TEA*C* declines, rate $TEAC \rightarrow KC$ becomes equal to and then smaller than rate $KC \rightarrow XC$, producing a peak in the current curve about 0.5 msec after the step. The subsequent decay of the current is considerably slower than for the control curves (Figs. 3 to 5), implying that the n^4 gate cannot close until the TEA$^+$ gate opens; i.e., until the channel is emptied of TEA$^+$. This is indicated in the arrow diagram by the absence of a direct path from TEA*C* to *XC*.

(*e*) In Fig. 4 *b* TEA*C* is initially small enough (TEA$^+$ below 1.2 mM) that the 0.5 msec peak is scarcely seen following a negative step to −97 mv from RP_{440K}. During the step to 82 mv, however, TEA*C* grows to a value large enough that a prominent peak is observed when V_m is again returned to −97 mv.

(*f*) In Fig. 7 of the previous paper (Armstrong and Binstock, 1965), it was noted that in 100 mM external [K$^+$], g_K reaches a plateau as V_m becomes more positive if TEA$^+$ is present inside the axon, but continues to increase in the absence of TEA$^+$. This can be explained by saying that as V_m becomes more positive n^4 gates tend to open but TEA$^+$ gates tend to close.

An attempt was made to fit this model to the data with the help of an analogue computer. The curves for positive steps could be fitted easily. The curves for negative steps could also be fitted, but a reasonable fit required the use of k greater than zero, which introduced a difficulty. If k is proportional to [TEA$^+$], and not equal to zero, the decline of the current for negative steps should become progressively slower as [TEA$^+$] increases; but experimentally the effect seems to saturate at [TEA$^+$] about 2.4 mM (Fig. 5). One possible remedy is to add another state, $TEAC_2$, to the right of TEA*C* in the arrow diagram, corresponding, perhaps, to a TEA$^+$ ion in the second site of a long pore. This alteration was not explored.

Despite the failure to fit the negative steps, it is of interest to approximate k for positive steps, and to indicate its usefulness. As discussed in (*c*) above, $KC \rightleftarrows TEAC$ seems to obey first-order kinetics, and its time constant, in terms of the present scheme therefore equals $1/(k + l)$. $1/\tau$ ($= k + l$) is plotted in Fig. 7 as a function of [TEA$^+$]. By hypothesis, k is zero when

$[TEA^+]$ is zero, so the zero $[TEA^+]$ intercept gives l. By hypothesis, l is independent of $[TEA^+]$. k is then $(1/\tau) - l$. Determined in this way, k is a linear function of $[TEA^+]$, indicating that only one TEA^+ ion is necessary to stop up a K^+ channel.

k is proportional to the rate at which TEA^+ ions enter the membrane. After making one assumption, k can be used to calculate the rate at which K^+ ions pass through the channels. The assumption is that at equal axoplasmic concentrations of TEA^+ and K^+, an ion entering a channel from the inside is

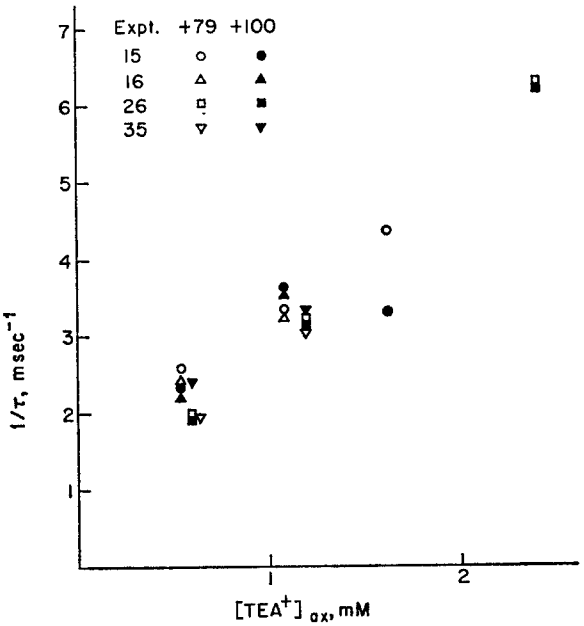

FIGURE 7. Plot of the reciprocal of the time constants from Table II as a function of the axoplasmic $[TEA^+]$.

equally likely to be a TEA^+ or a K^+; or generally, the fraction of the ions entering from the inside which are TEA^+ is

$$\frac{[TEA^+]}{[TEA^+] + [K^+]_{ax}}.$$

In the experiment of Fig. 5, the internal $[K^+]$ (estimated from RP_{440K} by means of the Nernst equation) is 250 mM. When $[TEA^+]$ is 2.6 mM, there is approximately one TEA^+ ion for 100 K^+ ions. Following a positive step applied at time zero, there is one chance in a hundred that the first ion entering a channel from the inside will be a TEA^+ ion; or, on the average, 0.99 of the initially open channels will be open after the first ion enters. After x ions have entered each channel, the fraction of the channels open will be

$0.99^x = e^{-0.01005x}$, provided that no TEA^+ ions have left the channels; i.e. if $l = 0$. But if $l = 0$, the current following a positive step would equal $I_\infty - (I_\infty - I_o)\, e^{-kt}$, which is the solution of the first-order system postulated, with $1/\tau = k$. Equating the logarithms of the two expressions (i.e. $-0.01005x = -kt$) and taking $k = 6$ from Fig. 7, $x = 600$ ions/msec. At this rate, 6.7×10^9 channels/cm^2 are necessary to account for the largest outward current in Fig. 5.

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