Interaction of Tetraethylammonium

Ion Derivatives with the

Potassium Channels of Giant Axons

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ABSTRACT   A number of compounds related to TEA^+ (tetraethylammonium ion) were injected into squid axons and their effects on g_K (the potassium conductance) were determined. In most of these ions a quaternary nitrogen is surrounded by three ethyl groups and a fourth group that is very hydrophobic. Several of the ions cause inactivation of g_K, a type of ionic gating that is not normally seen in squid axon; i.e., after depolarization g_K increases and then spontaneously decreases to a small fraction of its peak value even though the depolarization is maintained. Observations on the mechanism of this gating show that (a) QA (quaternary ammonium) ions only enter K^+ channels that have open activation gates (the normal permeability gates). (b) The activation gates of QA-occluded channels do not close readily. (c) Hyperpolarization helps to clear QA ions from the channels. (d) Raising the external K^+ concentration also helps to clear QA ions from the channels. Observations (c) and (d) strongly suggest that K^+ ions traverse the membrane by way of pores, and they cannot be explained by the usual type of carrier model. The data suggest that a K^+ pore has two distinct parts: a wide inner mouth that can accept a hydrated K^+ ion or a TEA^+-like ion, and a narrower portion that can accept a dehydrated or partially dehydrated K^+ ion, but not TEA^+.

INTRODUCTION

It is established beyond question that nerve membrane can distinguish between sodium and potassium ions, and that the permeability to these two ions is variable (Hodgkin and Huxley, 1952 a). How the membrane accomplishes this, or, for that matter, how the ions permeate the membrane are unanswered questions, though hypotheses on these matters are now almost too numerous to count. This paper provides experimental evidence on these questions, derived from a study of the interaction of quaternary ammonium (QA) compounds with the K^+ channels.

It has been reported that two types of ionic gating can be produced by injecting appropriate QA ions into squid axons. TEA^+ causes anomalous or ingoing rectification (Armstrong and Binstock, 1965), which is normally seen in muscle fibers (Katz, 1949; Noble, 1962), and pentyltriethylammonium ion (C_5) causes a rapid and rather incomplete inactivation of g_K , the potassium conductance (Armstrong, 1969); i.e. after depolarization g_K at first increases and then decreases, even though the depolarization is maintained. g_K inactivation with many of the same properties normally occurs in supramedullary neurons of the puffer fish (Nakajima, 1966). Inactivation of the sodium conductance (g_{Na}) is, of course, a normal property of many excitable membranes (Hodgkin and Huxley, 1952 b).

Though their effects on g_K are quite different, the mechanism of action of both TEA^+ and C_5 is basically the same. Both can enter and block only channels that have open activation gates, and both tend to be cleared from the channels by hyperpolarization. The difference in their effects on g_K arises from the relatively tighter binding of C_5 ions to the blocking sites, which are presumably in the channel mouths. The tighter binding of C_5 is apparently the result of hydrophobic bonding between the pentyl chain of C_5 and a hydrophobic region in or near the channel mouth. Since TEA^+ has an ethyl group in place of the pentyl group of C_5 , it binds less tightly to this hydrophobic region.

In order to characterize further the hydrophobic region of the channel mouth, the effects on g_K of a number of QA ions with very hydrophobic side chains have been examined and are reported here. Several of these bind very tightly to the blocking sites, and as a consequence cause almost complete inactivation of g_K . Observations on mechanism lead to several conclusions regarding the nature of the K^+ pathways through the membrane. Perhaps the most interesting conclusion is that the pathways cannot be simple carriers, but may well be pores.

METHODS

The experiments reported here were performed on segments of isolated axons from the Chilean squid *Dosidicus gigas*. The isolated axon segments were subjected to one or both of the following procedures, which have been described in more detail elsewhere (Armstrong, 1969).

Microinjection A thin glass cannula was pushed through the axoplasm for the full length of the axon segment. As the cannula was withdrawn, fixed amounts of an aqueous solution of the desired quaternary ammonium ion were injected through the cannula at 2–3–mm intervals. The total injected volume was usually about 1 μl into an axon segment of approximately 35 μl volume.

Most of the compounds tested have the general structural formula shown in Fig. 1. A central quaternary nitrogen is surrounded by three ethyl groups, and the fourth side

chain, R, is variable. The designations for the compounds used in this paper are given next to the various R's shown in the figure. For C_5, C_9, and C_{12}, R is an unbranched hydrocarbon chain containing 5, 9, or 12 carbons. Using this nomenclature, TEA$^+$ would be C_2. A benzene ring is represented by ϕ. Only one compound, trimethyl-octylammonium ion, was used which differed from this general plan. These compounds were prepared for me by Eastman Kodak Co., Rochester, N. Y. For microinjection, an aqueous solution of one of the compounds was prepared, at a concentration that was about 35 times the intended axoplasmic concentration. As a guess, the actual concentration in the axoplasm was within 50 % of the intended value.

Voltage Clamp In order to control, or clamp, the membrane potential (V), a platinum-blacked wire was inserted through the length of the axon segment from one
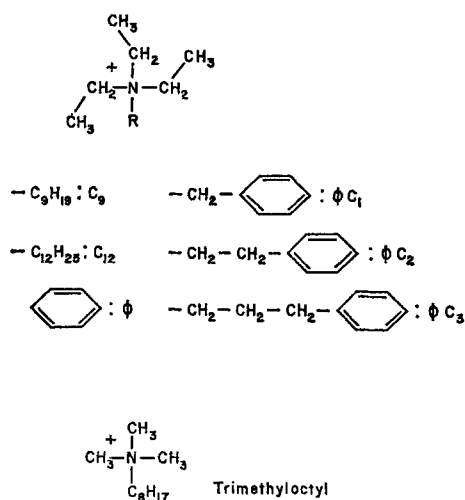

FIGURE 1. Structural formulae of the quaternary ammonium ions used. The R groups are shown with the abbreviation used to denote them.

end, and a low impedance pipette was inserted from the other end to measure the potential inside the axon at the center of the segment. An external electrode measured the potential outside the axon, and except for errors caused by the series resistance, the difference in potential recorded by the internal and external electrodes is V, the membrane potential. V was controlled by a feedback circuit, which passed the necessary current between the internal platinum-blacked electrode and external current electrodes (which were separate from the external potential electrode). The performance of the control circuit has been described elsewhere (Armstrong, 1969). The errors caused by the series resistance were usually fully compensated for by feedback of a signal proportional to the current density.

Following the usual convention, V in this paper is the potential inside the axon minus the potential outside. A depolarization makes V more positive, and repolarization in most cases returns V to the holding potential, which is the V that the clamp circuit is set to maintain between pulses. The traces in most figures show the current

necessary to maintain the potential at the level commanded. Outward current is considered positive, and in all figures is up, and inward current is negative and down. The terms sodium conductance (g_{Na}) and potassium conductance (g_K) are used as they were defined by Hodgkin and Huxley (1952 a), namely,

$$g_{Na} = I_{Na} (V - V_{Na})$$

$$g_K = I_K (V - V_K)$$

where V_{Na} and V_K are the equilibrium potentials for sodium ion and for potassium ion, and I_{Na} and I_K are the currents through the membrane of these ions.

The temperature was usually maintained at 8°-10°C by continual flow through the chamber of chilled external solution. Three external solutions were used, and their names and compositions were as follows:

ASW: Na+, 430 mM; K+, 10 mM; Ca++, 50 mM; Cl-, 560 mM.

112K: Na+, 323 mM; K+, 112 mM; Ca++, 10 mM; Mg++, 50 mM; Cl-, 555 mM.

440K: K+, 440 mM; Ca++, 10 mM; Mg++, 50 mM; Cl-, 560 mM.

External solutions were buffered with Tris [tris(hydroxymethyl)aminomethane], 2 mM, to a pH of about 7.5.

RESULTS

When a step depolarization is applied to a normal axon under voltage control, the potassium current (I_K) begins to increase after a short delay, at a rate which is faster the larger the depolarization. After some milliseconds, I_K reaches a fairly steady level that is maintained for the duration of the depolarization, provided it is not too long. Fig. 2 d shows this normal I_K pattern in a fiber that has been poisoned with 10⁻⁷ M tetrodotoxin (TTX), which selectively eliminates the sodium current, I_{Na} (Moore and Narahashi, 1967; Hille, 1968). The currents for a number of depolarizations are superimposed in the frame, and V for each trace is indicated. Before each depolarization, V was held at −60 mv for at least 2 sec.

This pattern is drastically changed by injection into the axoplasm of any one of several QA ions. Fig. 2 b shows the currents of a TTX-poisoned axon containing one of these QA ions (C₉) at an axoplasmic concentration of 0.11 mM. I_K rises after a delay, much as it does in a normal axon, but after 2–5 msec, depending on the depolarization, it declines (inactivates) to a small fraction of its peak value, even though the depolarization is maintained. 15 msec after the beginning of the depolarization, V was returned to −60 mv. The membrane conductance (g_m) at the instant of repolarization can be calculated by dividing the change in membrane current by the change in V, and even without calculation the small size of the current change shows that g_m has fallen to a low value. Further evidence on this point is given below.

Fig. 2 *a* shows the currents of the same C_9-containing axon before the addition of TTX. Very soon after depolarization there is a current of quickly changing magnitude that is inward for all except the two largest depolarizations. This current is carried predominantly by sodium ion, and it is outward when V is more positive than V_{Na}, the equilibrium potential for sodium ion. I_{Na} in Fig. 2 *a* rises to a peak and rapidly inactivates, as it does in a normal
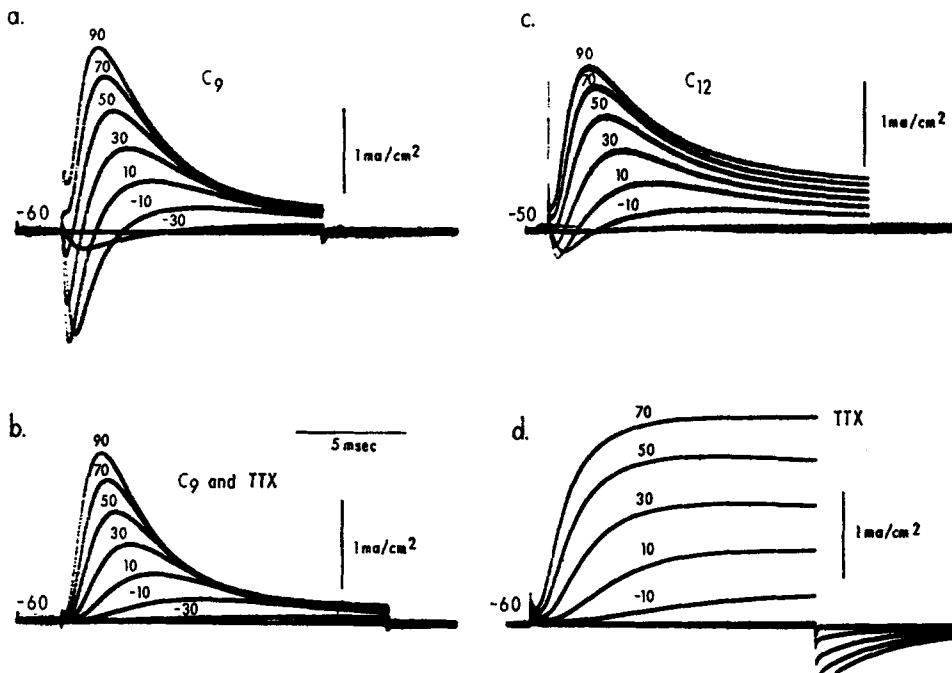

FIGURE 2. Membrane currents of an uninjected axon (*d*), and of axons containing C_9 or C_{12}. The axons were in ASW, to which 10^{-7} M TTX was added in (*b*) and (*d*). Membrane potential is given by the numeral near each trace. (*a*) and (*b*) are from the same axon, which had 0.11 mM C_9 in the axoplasm; 10°C. (*c*) Another axon, with 0.2 mM C_{12} in the axoplasm. The holding potential was −50 mv, and I_{Na} is consequently small; 9°C. (*d*) Another axon, not injected.

axon (Hodgkin and Huxley, 1952 *b*). QA ions cause no obvious changes in the properties of I_{Na}, though the matter was not studied in detail. In general, I_{Na} and I_K seem to respond to QA ions in a completely independent manner, as though the currents of these two ions are conducted through the membrane by two separate sets of channels (see Hille, 1970; Rojas and Armstrong, 1971).

In the experiment of Fig. 2 *c*, the axon contained 0.2 mM C_{12}. No TTX was present, but V was held at a low value (−50 mv) to inactivate most of g_{Na}, and I_{Na} is consequently small. I_K rises after depolarization and then inactivates but inactivation at this concentration of C_{12} may be somewhat less complete

than with C_9. Very little can be said quantitatively about the relative effectiveness of C_9 and C_{12} without further experimentation.

In terms of the equation for I_K (or g_K) given in Methods, I_K can diminish as a result of either a decrease in g_K or a decrease in V-V_K. Since V is held constant, the latter explanation would require that V_K, the equilibrium potential for potassium ion, changed. The experiment in Fig. 3 a was performed to
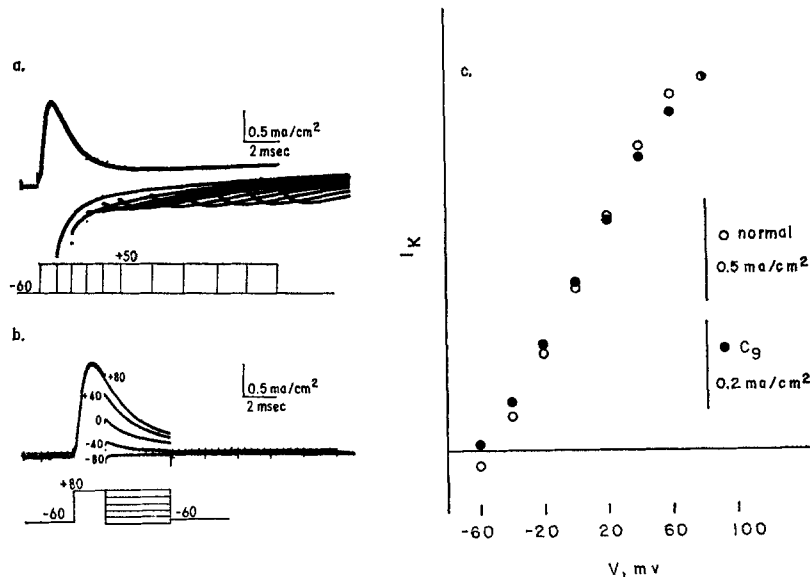

FIGURE 3. (a) Currents for depolarizations of various durations, showing that membrane conductance declines in proportion to outward current. The axon was in 440K and contained 0.11 mM C_9 ; 11°C. (b) Determination of the "instantaneous" current voltage curve, which is plotted in (c). The axon was in ASW and contained 0.1 mM C_9; 9°C. (c) "Instantaneous" I-V curve of the axon illustrated in Fig. 3 b, and the almost indistinguishable curve of a normal axon.

distinguish between these possibilities. The axon was bathed in 440K, and had 0.11 mM C_9 in the axoplasm. Before depolarizing to +50 mv, V was held at −60 mv for about 1 sec (resting potential was +4 mv). After 1, 2, or more msec at +90, V was returned to −60, producing a transient inward current. g_m is proportional to the difference between the current just before and just after repolarization and, because there is no I_{Na}, g_m is approximately equal to g_K. From the figure it is clear that I_K and g_K during a maintained depolarization are proportional and that I_K declines as a result of a decrease in g_K.

In Fig. 3 a, when V is returned to −60 mv after a period of depolarization, there is a transient inward current whose shape depends on the duration of the depolarization. This current is carried predominantly by potassium ion, and it is inward because in 440K the potassium equilibrium potential is near zero,

and $V\text{-}V_K$ is negative for $V = -60$ mv. After a 1 msec depolarization the inward I_K is initially large, and it declines monotonically in the way described by Hodgkin and Huxley (1952 a) as g_K turns off. After depolarizations of more than 3 msec duration the pattern is completely different. I_K (inward) is initially small and it increases in magnitude before decreasing. An explanation for this pattern is given in the Discussion.

It has been postulated (Armstrong, 1969) that the effect of QA ions on a single channel is all or none: either the channel is occluded, or it conducts
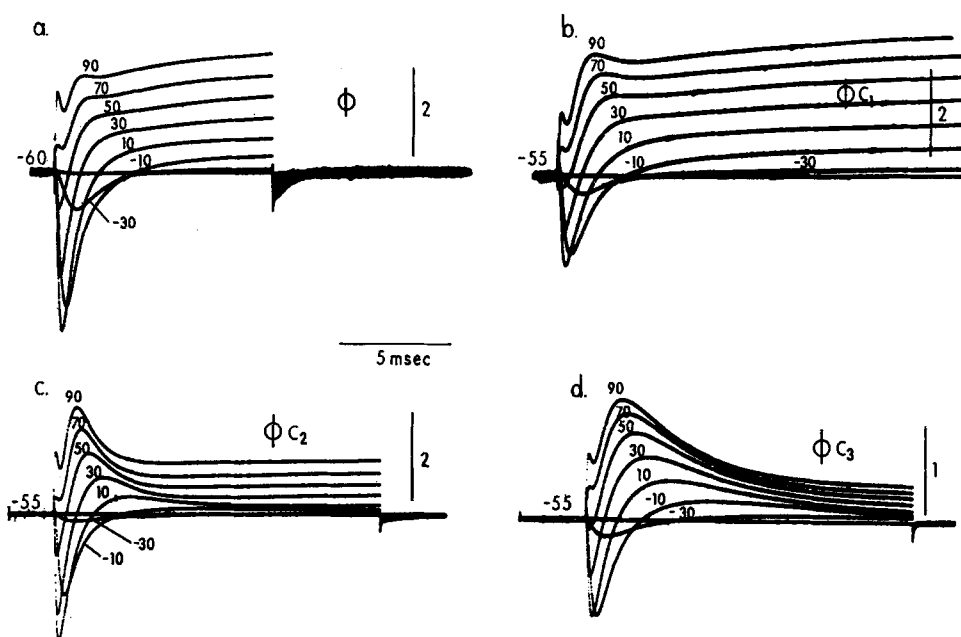

FIGURE 4. Membrane currents of four axons in ASW containing analogues which have benzene rings in one of the four side chains. (a) 0.2 mM ϕ, 13.8°C. (b) 0.15 mM ϕC_1, 12.3°C. (c) 0.2 mM ϕC_2, 14.5°C. (d) 0.09 mM ϕC_3, 12.8°C. The vertical calibrations give current, in milliamperes per square centimeter.

normally. If this is so, the instantaneous $I\text{-}V$ curve of the unoccluded channels should have the same shape as in the normal axon. Fig. 3 b shows a determination of this curve in a C_9-injected axon. The fiber (which was in ASW) was depolarized to $V = +90$ for 2 msec, and V was then changed to the values given in the figure. In Fig. 3 c, the current immediately after the second potential step is plotted as a function of V during the second step, and it can be seen that the curve has the same shape as the curve of a normal axon, which is given for comparison.

The compounds with benzene rings in the R chain form an interesting series, as shown in Fig. 4. The compounds ϕ and ϕC_1 cause very little inactiva-

tion, but there is a peak in the current curve for large depolarizations that shows they are not altogether without effect. Addition of one more carbon between the benzene ring and the nitrogen, to form ϕC_2, increases blocking effectiveness greatly, and yet another carbon, to make ϕC_3, produces a compound that is as effective, by this test, as C_9 or C_{12}. It will be shown below that ϕC_3 binds to the blocking site even more tightly than C_9. If one compares the series in which R is a straight hydrocarbon chain with the benzene ring series, it is clear that not only the number of carbons in R is important, but also their arrangement. ϕ and ϕC_1 are relatively ineffective, but the addition of three or two methyl groups to make ϕC_3 produces a very effective blocker. Three methyl groups added to TEA+, to make C_5, on the other hand, have much less effect on blocking potency.

Preliminary experiments were performed with another compound that had differences other than in the R group. This was trimethyloctylammonium ion, a quaternary nitrogen surrounded by three methyl groups and one straight chain octyl group (Fig. 1). The data above show that this compound is well equipped with regard to the R group (the octyl chain) to be a potent I_K inhibitor. At concentrations of approximately 0.3 mM and 1.2 mM, however, the compound does not cause detectable inactivation. The absence of effect suggests that the compound cannot enter the blocking sites.

Rate of Inactivation

The rate at which I_K inactivates on depolarization depends on several factors.

(a) Inactivation rate increases and peak I_K is depressed as the QA concentration in the axon is increased. This is illustrated in Fig. 5, which shows, superimposed, I_K from a single axon for [C_9] equal to 0.058 mM and 0.21 mM. Notice that shortly after depolarization I_K increases at about the same rate at both concentrations.

(b) Inactivation rate depends on V, and this dependency can in turn be split into two factors. (i) Inactivation occurs more rapidly for large depolarization, in part because g_K activates more rapidly under these conditions, and activation of g_K must precede inactivation (Armstrong, 1969). This can best be seen by means of the experiment used by Hodgkin and Huxley (1952 b) to study the time course of g_{Na} inactivation: a small conditioning depolarization is applied to the membrane, and after a variable interval a test pulse is applied. (See diagram, Fig. 6, which illustrates the pulse sequence for a conditioning pulse of 8 msec.) The maximum amplitude of the current during the test step gives a good, though not perfect, estimate of the fraction of g_K that is still *not* inactivated, and hence an estimate of the inactivated fraction. The rate of inactivation is proportional to g_K (or at constant V, to I_K), as can be seen in Figs. 6 and 7. In Fig. 6, inactivation scarcely occurs during the first 2 msec of the conditioning pulse, when I_K has not yet turned on. Later, the peaks decline

most steeply when I_K of the conditioning step (the lower, continuous trace) has reached its maximum value. This proportionality is shown more clearly in Fig. 7. I_K immediately after application of the test step is given by the ×'s, while the filled circles give the negative of the change in peak height on an interval divided by the length of the interval. The rate of change of peak height has been scaled arbitrarily, and differently in each part of the figure, to match the I_K points. From these plots it is evident that inactivation rate and g_K are proportional during the first few milliseconds after depolarization. At longer times one must take into account the rate of reactivation as C_9 ions dissociate from the blocking sites. If the inactivated fraction of $\bar{g}_K$ ($\bar{g}_K$, as defined by Hodgkin and Huxley, 1952 a, is equal to g_K when all channels are
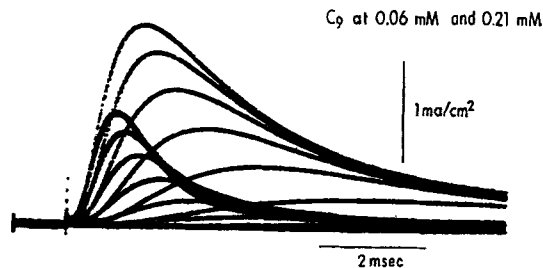

FIGURE 5. The effect of concentration on inactivation rate. Both sets of currents are for depolarizations to −30 mv through +90 mv in 20-mv steps, from a holding potential of −60 mv. The higher amplitude curves are for 0.06 mM axoplasmic C_9, and the lower curves are from the same axon after a second injection raised the C_9 concentration to 0.21 mM; 9°C.

conducting) is called z, and the conducting fraction is called y ($= g_K/\bar{g}_K$), the complete formula is (see Armstrong, 1969)

$$\frac{dz}{dt} = \kappa \cdot y - l \cdot z.$$

Translated into experimental quantities, this is

$$\frac{\Delta z}{\Delta t} = \kappa \cdot \left(\frac{I_{K_{avg}}}{\bar{I}_K}\right) - l \cdot z_{avg}.$$

In this formula, I_K and $\bar{I}_K$ have been substituted for, respectively, $V \cdot g_K$ and $V \cdot \bar{g}_K$, and the subscript avg means the average value of the variable on the interval. $\bar{g}_K$ (or $\bar{I}_K$) was not determined in this experiment, and it is consequently impossible to know the absolute values of y and κ, but l can be determined explicitly. The open circles in the figure were calculated by this formula, using $\kappa/\bar{I}_K = 0.22$ msec⁻¹, $l = 0.026$ msec⁻¹ for $V = -30$; $\kappa/\bar{I}_K = 0.25$ msec⁻¹, $l = 0.026$ msec⁻¹ for $V = -20$; and $\kappa/\bar{I}_K = 0.27$, $l = 0.032$ msec⁻¹

for $V = -10$. The calculation, of course, is not very accurate because the computing interval is large. (ii) The other way in which inactivation rate de-
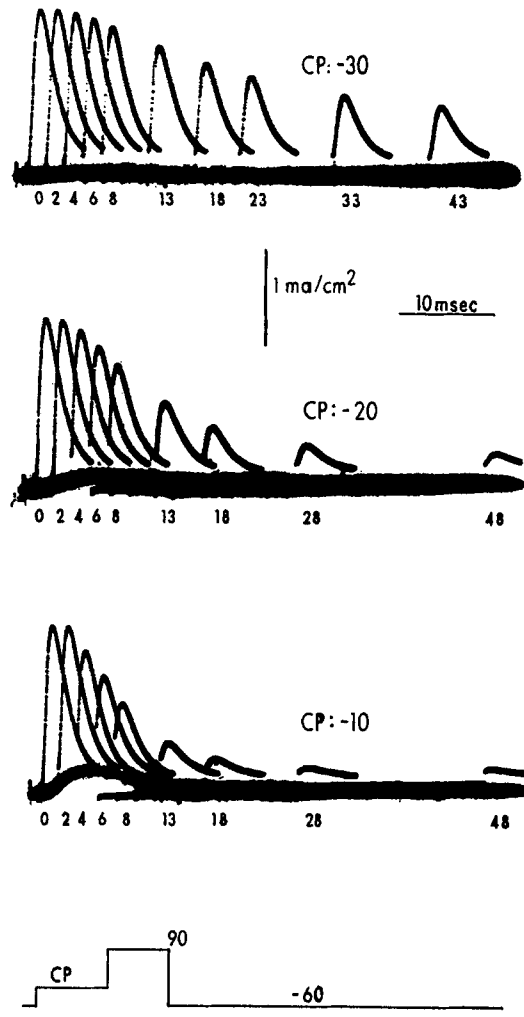

FIGURE 6.   The effect of membrane potential on inactivation rate. The voltage diagram gives the step sequence for the peaks labeled 8, which means that the test depolarization (to 90 mv) was preceded by a conditioning pulse (CP) of 8 msec duration. Membrane potential during the conditioning pulse is given for each frame. Inactivation does not begin until g_K becomes appreciable; 10.5°C.

pends on V is that κ, the proportionality factor between g_K and inactivation rate, may change with V, or V-V_K . There is little evidence on this at present.

(c) At a given concentration, inactivation rate probably depends on the QA ion employed. Evidence was given above to show that trimethyloctylammo-

nium, for example, enters the blocking sites slowly, if at all. Probably entry rate varies within the triethyl-R ammonium group, but the variation must be fairly small and can be detected with certainty only in internal perfusion experiments, in which the QA concentration can be known with accuracy.
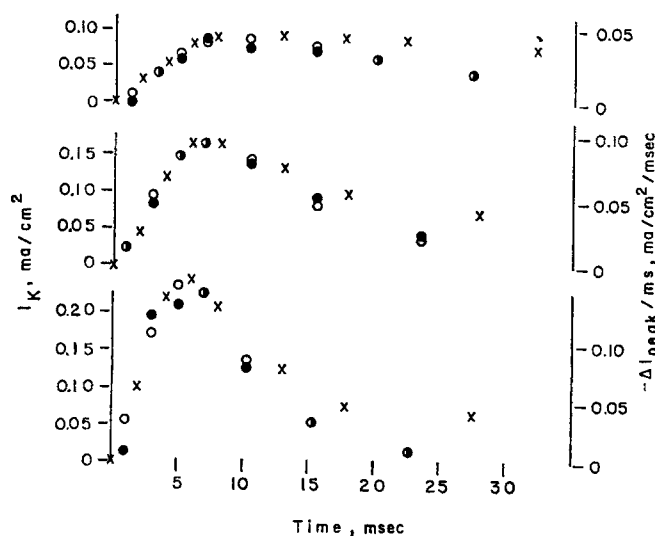

FIGURE 7.   The proportionality between inactivation rate and I_K. x, I_K from Fig. 6. ●, a rough measure of the slope of the envelope curve of the peaks in Fig. 6. The negative of the change in peak height on an interval has been divided by the length of the interval, and scaled to match the I_K curve. O, expected rates of peak height change, as calculated from the formula in the text.

Rate of Recovery from Inactivation

When the membrane is repolarized after some milliseconds of depolarization, g_K recovers from inactivation as QA ions leave the blocking sites. The procedure for following recovery is diagrammed in Fig. 8. Inactivation is produced by a first depolarization to 90 mv. After 10 msec, the membrane is repolarized (usually to −60 or −100) for a time and a second depolarizing pulse to 90 mv then tests the extent of recovery during the period of repolarization. Like the rate of inactivation, recovery rate depends on a number of factors, and this constitutes a subject of great interest.

(a) Recovery rate depends on the QA ion used to produce inactivation. The half-time of recovery for a number of compounds is shown in Table I. (The half-time is that time required to recover half the difference between the highest and lowest values of the current.) All of the figures shown are for recovery at $V = -60$ mv. From comparing half-time measurements for which temperature and concentration were about the same, one can see that re-

covery took by far the longest when ϕC_3 was the inactivating substance. For C_9 and C_{12}, recovery was much faster. Two substances that cause less complete inactivation, ϕC_1 and ϕC_2 (see Fig. 4), have shorter, or, for ϕC_1, much shorter, recovery time. In general it seems that potent inactivators have long recovery times, but the single experiment with C_5 is an exception to the rule since it does not cause very pronounced inactivation, but has a rather long recovery time. It must be mentioned that recovery is a complicated process,
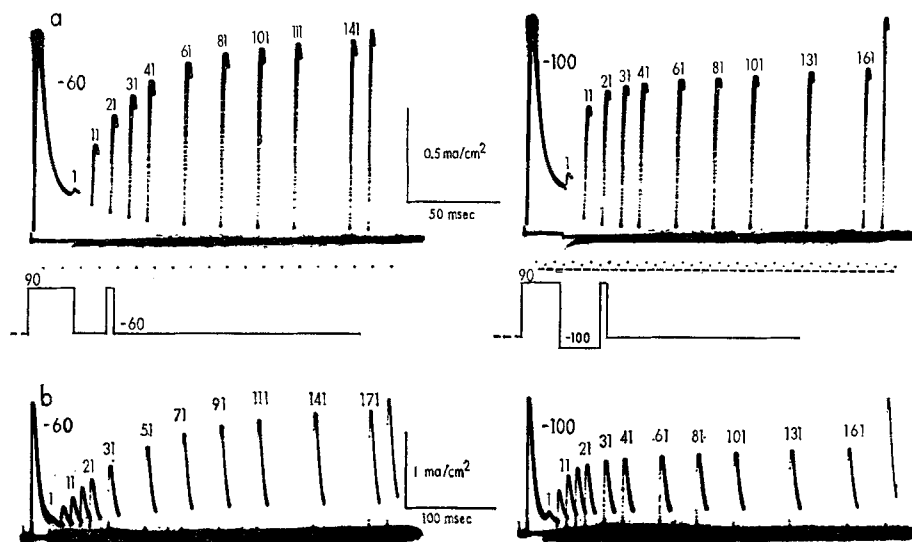

FIGURE 8. Recovery from inactivation of two C_9-containing axons, and the effect of hyperpolarization. The procedure for the current peak marked 21 is shown in the potential diagram. The numbers over the current peaks give the duration of the interval between the end of the conditioning pulse (to +90 mv) and the beginning of the test step (to +90 mv). (a) Axoplasmic C_9 concentration was 0.04 mM; 9°C. (b) Another axon, containing 0.11 mM C_9; 10.5°C.

and half-time variations can be the result of a change in any of several factors, as discussed below.

(b) Recovery rate depends on V in a complicated and interesting way. At $V = -60$ (Fig. 8) the amplitude of the test peaks at first increases fairly rapidly, and then, at a slower rate, it climbs steadily toward the control amplitude (the first and last peaks in the frame). At $V = -100$, the initial phase of recovery is considerably faster than at -60, but the later phase is slowed: peak height reaches a plateau, and full recovery requires several seconds. The current peaks during the plateau are more depressed when the C_9 concentration is higher, as can be seen by comparing Fig. 8 a (0.4 mM) with Fig. 8 b (0.11 mM). Hyperpolarization thus has two effects on recovery, speeding the early phase and slowing the later phase. Slowing of the later phase will be

called the trapping effect, for reasons that will be clear in the Discussion. This is unlike recovery from g_{Na} inactivation, which is simply speeded by hyperpolarization (Hodgkin and Huxley, 1952 b).

TABLE I
RECOVERY HALF-TIMES

Compound	Concentration	External Solution	Temperature	V_{Rec}	$t_{1/2}$
	mM		$°C$	mv	$msec$
C$_5$	0.27	ASW	10	−60	23⎫
	0.27	"	18	−60	6⎭
C$_9$	0.10	ASW	11	−60	30⎫
	0.10	"	6	−60	77⎬
	0.10	112K	8	−60	36⎭
	0.12	ASW	9	−60	43⎭
	0.11	ASW	9	−60	38⎫
	0.11	440K	10	−60	6⎭
	0.06	ASW	8	−60	38⎫
	0.21	"	10	−60	77⎭
	0.04	ASW	8	−60	21⎭
C$_{12}$	0.20	ASW	7	−55	55⎭
	0.16	ASW	8	−60	45⎭
ϕC$_1$	0.15	ASW	13	−55	<2
ϕC$_2$	0.23	ASW	11	−55	42⎫
	0.23	"	15	−55	18⎭
ϕC$_3$	0.18	ASW	11	−55	250⎫
	0.18	440K	11	−55	57⎭
	0.11	ASW	11	−60	200⎫
	0.11	112K	10	−60	90⎬
	0.11	440K	10	−60	50⎭
	0.09	ASW	11	−60	160⎫
	0.26	"	11	−60	220⎭

V_{Rec} , membrane potential during recovery.
$t_{1/2}$, half-time of recovery.
Determinations from a given axon are grouped together.

Recovery rate after repolarization is proportional to g_K . The time course of g_K can be followed by looking at the initial value of the current produced by the test steps (Fig. 8 a). At $V = -60$, g_K declines monotonically with a half-time of about 21 msec, and at -100 the half-time is about 5 msec. At both voltages, the rapid phase of recovery ends when g_K has fallen to a low value,
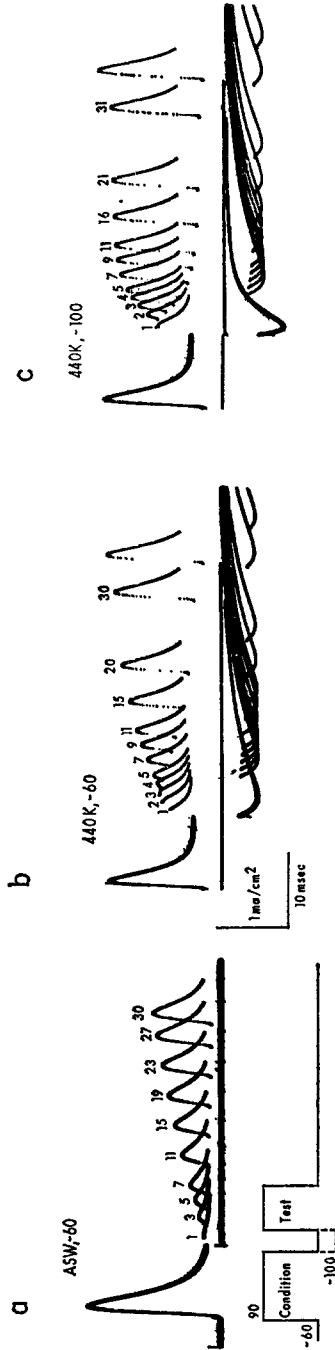
FIGURE 9. The effect of raising external K$^+$ on the rate of recovery from inactivation. Same procedure as in Fig. 8, but at faster sweep speed; 10.5°C. This is the same axon as in Fig. 2 a and b and Fig. 8 b.
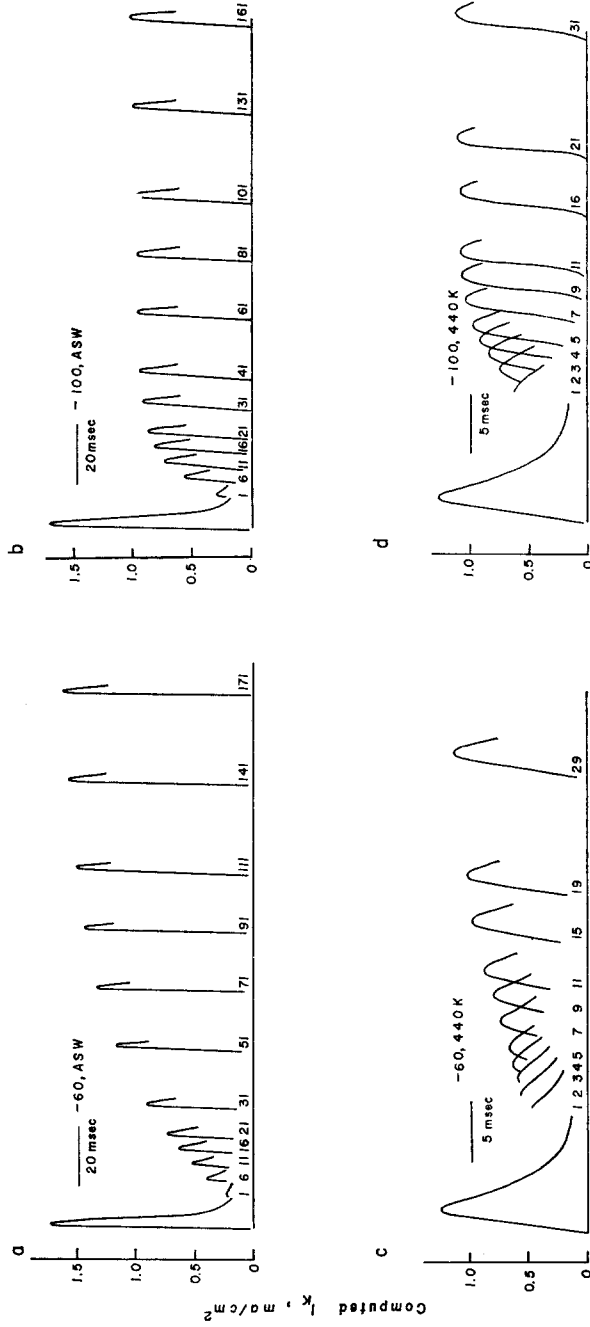
FIGURE 10. Theoretical recovery curves, calculated from the model discussed in the text, using the constants given in Table II. (a), (b), (c), and (d) resemble, respectively, Fig. 8 b, −60; Fig. 8 b, −100; Fig. 9, 440K, −60; and Fig. 9, 440K, −100.

and at -60 in particular the proportionality between g_K and recovery rate is unmistakable.

The fall of g_K on repolarization is much slower in the presence of a QA compound than in its absence, if the compound is an effective g_K inhibitor. In a normal axon, g_K falls with a half-time (at $10.5°C$) of 2.3 msec at $V = -60$, and approximately 0.7 msec at -100, much faster at both voltages than in Fig. 8.

A final point about the decline of g_K is that it is not always monotonic when QA ions are present. On repolarization g_K was found to increase initially, and then decrease, when C_5 was present (Armstrong, 1969). The same phenomenon is observed when C_9 is present at higher concentration (not illustrated) or when $[K]_{out}$ is elevated (Fig. 9 b). An explanation for the various effects of hyperpolarization will be given in the Discussion.

(c) Raising the external K^+ concentration greatly speeds recovery. Fig. 9 shows the recovery of an axon in ASW and in 440K, in both cases at $V = -60$, temperature about $10.5°C$, and with 0.11 mM C_9. In 440K, the half-time of recovery is much smaller, 6 msec as against 36 msec in ASW, and there are interesting changes in the current transients produced by the first few test pulses. Compare first the test pulses 1 msec after repolarization. In ASW, g_K at the beginning of the test pulse is about the same as at the end of the conditioning pulse, while in 440K it jumps by a factor of almost three, and falls monotonically during the test pulse. Only after several milliseconds of recovery do the test currents in 440K show an initial rise in amplitude. From the shortened recovery time, it is clear that raising external K^+ hastens dissociation of C_9 from the blocking site (see Table I for other examples) and this has significant implications which are discussed below.

(d) From the limited data in Table I, increasing the temperature cuts recovery half-time with a Q_{10} of about 6 for C_9. As pointed out above, however, recovery is a complex process, and half-time can be affected by changes in several rate constants.

It should be mentioned that in axons not poisoned by TTX it was possible to monitor simultaneously the recovery from inactivation of both g_{Na} and g_K, and the results again point to the complete independence of the two conductances. In an axon containing C_9, for example, g_{Na} recovers with a normal time course, and is completely restored after a few milliseconds of repolarization, at a time when g_K has scarcely begun recovery.

Ineffectiveness of External C_9

0.2 mM C_9 applied to the outside of an axon has no effect on the currents unless the axon is left immersed in the C_9-containing medium for more than half an hour. After a prolonged soak (40 min at $12°-20°C$) there was, in one experi-

ment, detectable inactivation of g_K, but much less than is produced by 0.1 mM C_9 inside the axon.

0.2 mM C_{12} applied externally caused detectable inactivation within 5 min after application (no measurements were made at shorter times) but much less than is produced by an equal concentration in the axoplasm.

From these experiments it seems clear that QA ions have access to the outer membrane surface (the C_{12} experiment) but they must be inside the axon to affect I_K. C_9 apparently is very sparingly permeable, and therefore accumulates in the axoplasm very slowly. C_{12}, being more lipid soluble, permeates more rapidly.

DISCUSSION

The main finding of this paper is that inactivation gates for the potassium channels can be constructed simply by injecting an appropriate quaternary ammonium ion into the axoplasm. The remainder of the paper is devoted to developing a kinetic model that can reproduce the experimental findings, and determining what the model tells about the mechanism of ion permeation and gating mechanisms. Since the discussion is long, it may be useful to begin with a summary of the conclusions. First, a kinetic model is proposed which has as its most essential features (a) QA ions can enter the blocking sites only when the activation, or n^4, gates of the channels are open, and (b) the activation gate of a QA-occluded channel does not close readily. In the terms of this model, the effect of making the R group more hydrophobic (see Fig. 1) is to diminish the rate constant for dissociation of the QA-blocking site complex. This dissociation rate is enhanced by increasing the external K^+ concentration, and this fact can only be explained by postulating that the blocking site is directly in the K^+ channel. It is concluded that the data are incompatible with a carrier model for passive ion transport.

Kinetic Model

The state of the activation (or n^4) gate of a potassium channel can be described very simply:

$$\text{closed} \rightleftarrows \text{open}.$$
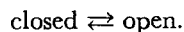
The kinetics of the transition between the two states were given by Hodgkin and Huxley (1952 a) and are not first order. To account for the effects of TEA^+ and C_5 on g_K, these kinetics were assumed to be unaltered, and an additional state was added (Armstrong, 1966, 1969):

$$\text{closed} \rightleftarrows \text{open} \underset{l}{\overset{\kappa}{\rightleftarrows}} \text{inactivated}.$$
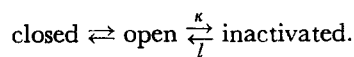
The last step, open to inactivated, has first-order kinetics, and corresponds to a QA ion entering a site that blocks the channel. The arrangement of the compartments has two implications that may not be immediately obvious. First, a channel cannot inactivate unless it is open; second, the activation gate of the channel cannot close if a QA ion is in the channel. (The second statement requires modification, as shown below.) This scheme will account for many but not all of the observations reported above, and these will be discussed one by one.

(*a*) More-or-less complete inactivation. As shown previously (Armstrong, 1969) inactivation can in theory be made more complete by decreasing the rate constant l (see arrow diagram above) and less complete by increasing it. Or, κ can be increased, but this depresses peak current as well as steady-state current. Experimentally, l can be decreased with little effect on κ by making group R more hydrophobic, and this is presumably because R binds to a hydrophobic moiety associated with the blocking site. The experiments with ϕ, ϕC_1, ϕC_2, and ϕC_3 suggest that the hydrophobic part of the blocking site is separated by several Ångstroms from the polar part to which the triethyl-N portion of the QA ion binds.

(*b*) The increase of inactivation rate with increase of QA concentration. This is simply explained in theory by letting κ increase with QA concentration. Experiments indicate that κ is probably a linear function of QA concentration, as it would be if a one QA–one channel complex were formed (Armstrong, 1966; Hille, 1967).

(*c*) Proportionality of inactivation rate and g_K. This follows directly from the scheme, since the inactivation rate is proportional to the number of open channels (see Results).

(*d*) Correlation between completeness of inactivation and slowness of recovery. Recovery is governed by the rate of disassociation of the QA-blocking site complex, and this rate is given by the product $l \cdot z$, where z is the fraction of the channels that are inactivated. The ratio open:inactivated is given by $l/(\kappa + l)$. Thus, as l increases, inactivation becomes less complete, and recovery becomes faster.

(*e*) The delayed fall of g_K in the presence of QA ions. On repolarization, channels progress from the inactivated state to open to closed. The channel population in the open state during recovery is buffered by inactivated channels that are opening as they lose QA ions.

(*f*) Proportionality between g_K and recovery rate. The scheme provides only one recovery path: inactivated channels must open, and cannot, for example, pass directly from the inactivated to the closed state. The number of open channels depends, in part, on the rate at which channels pass from inactivated to open, and the observed proportionality follows from this.
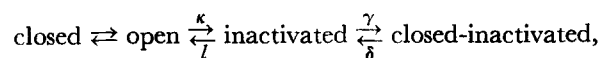
(*g*) The changing time course of the inward currents on repolarization (see

Fig. 3 *a*). g_K on repolarization normally decreases as the activation gates close, but it may at first increase in QA-containing axons because QA ions are leaving inactivated channels faster than open channels are closing. The rate of closing of open channels is proportional to the fraction of the channels that are open (fraction open multiplied by 4β; see below), and the rate at which QA ions leave inactivated channels is proportional to the fraction of the channels that are inactivated, i.e., the rate of leaving is $l \cdot z$. During a maintained depolarization, z increases as the open fraction decreases, and the rate at which QA ions leave the channels may thus come to exceed the rate at which open channels are closing.

The scheme above will not reproduce the effect of hyperpolarization on recovery rate. Enhancement of the early phase of recovery by hyperpolarization could be accounted for by increasing l, but one is left with no explanation for the slow later phase. This leads to the following question.
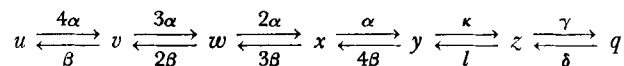
Can the Activation Gates Close When QA Ions Are in the Channels?

The answer assumed in the scheme above is no, but this answer must now be modified to say instead that the activation gates of QA-filled channels can close, but with difficulty. The evidence that QA-filled channels do not close readily is that, on repolarization, g_K falls much more slowly in a QA-containing axon than it does in a normal axon. If the gates of QA-filled channels closed at the normal rate, then g_K would fall at the normal rate despite the presence of QA ions. Further, at $V = -60$ mv, the time course of recovery from inactivation can almost be reproduced by the scheme given above, which does not include a closed-inactivated state, so at -60 mv closing of the activation gates of QA-filled channels must not occur to a great extent. At $V = -100$ mv, however, the scheme above is clearly inadequate, and unable to explain the dual effect of hyperpolarization on recovery rate. In order to account for the effects of hyperpolarization, one can postulate that the activation gate of a QA-blocked channel can close, but that it does so very slowly at $V = -60$, more readily but still slowly at $V = -100$. An arrow diagram for this is

$$\text{closed} \rightleftarrows \text{open} \underset{l}{\overset{\kappa}{\rightleftarrows}} \text{inactivated} \underset{\delta}{\overset{\gamma}{\rightleftarrows}} \text{closed-inactivated},$$

where closed-inactivated is the state of a channel that is QA-occluded and has its activation gate closed. The physical significance assigned here seems reasonable, because it is known that hyperpolarization hastens the closing of the n^4 gates. Formally, however, all that is needed is an additional compartment, and its physical meaning need not be specified. The workings of this expanded scheme can best be illustrated by a calculation, as in Fig. 10. It was necessary to use information from several different axons, and the calculated curves are thus at best good but not perfect simulations of Figs. 8 and 9.

The method of calculation is in most respects the same as in a previous paper (Armstrong, 1969). The kinetic scheme

$$u \underset{\beta}{\overset{4\alpha}{\rightleftarrows}} v \underset{2\beta}{\overset{3\alpha}{\rightleftarrows}} w \underset{3\beta}{\overset{2\alpha}{\rightleftarrows}} x \underset{4\beta}{\overset{\alpha}{\rightleftarrows}} y \underset{l}{\overset{\kappa}{\rightleftarrows}} z \underset{\delta}{\overset{\gamma}{\rightleftarrows}} q$$

was described by a set of seven simultaneous linear first-order differential equations, which were solved numerically on a PDP 12 digital computer. Compartments u, v, w, and x correspond to channels with closed activation gates, and, respectively, 4, 3, 2, and 1 of the hypothetical blocking particles of Hodgkin and Huxley (1952 a) in blocking position. A channel in state y has an open activation gate and is conducting. In state z the activation gate is open, but the channel is QA occluded. In state q, the activation gate is closed and the channel is QA occluded.

The constants of the scheme were determined as follows: α and β are the same as the α_n and β_n of Hodgkin and Huxley (1952 a) and are determined from the kinetics of g_K of a normal axon. κ at $V = +90$ was determined from an unpublished internal perfusion experiment. After setting κ at $V = +90$, $\bar{g}_K$ was adjusted to obtain the peak amplitude seen in Fig. 8, and l was determined from the final level of I_K. In order to limit the number of adjustable constants, κ at $V = -60$ was estimated from the $\kappa/\bar{g}_K$ values of Fig. 7, by extrapolation to -60. Because of the uncertainty of this estimate, calculations were done with several values of κ.

Having estimated γ, β, $\bar{g}_K$, and κ as just described, l, γ, and δ were determined by fitting the scheme as closely as possible to Figs. 8 and 9. Roughly speaking, l governs the rapid phase of recovery, γ determines the initial height of the plateau, and δ determines the rate of recovery during the slow phase. The range of values of l, γ, and δ which gave a reasonable facsimile of Figs. 8 and 9 was quite narrow.

Fig. 10 shows the results of several calculations with this model. The values of the constants used are given in Table II and are marked by double-daggers. Also given are other sets of constants that give fits (not illustrated) of about the same quality. The calculations reproduce quite well the features of Figs. 8 and 9. In particular the dual effect of hyperpolarization is accurately reproduced, as well as the effect of high external K+ on recovery rate, and the shape of the current transients during the second pulse in the 440K experiments.

Several firm conclusions can be drawn from the calculations. (a) The delayed fall of g_K on return $V = -60$, and the small size of γ required in fitting make it certain that the activation gates of QA-occluded channels close very reluctantly at this voltage. Possibly the activation gates of occluded channels can close at -100, but it must be admitted that the trapping effect conceivably arises in some other way. (b) To reproduce the effects of increased external K+, it is sufficient to raise l by a factor of four or five. Since l is the rate constant for dissociation of QA ions from the blocking sites, the physical meaning of this is that raising external K+ hastens dissociation of the QA–blocking site complex. (c) Hyperpolarization hastens dissociation, particularly if external K+ is high. From Table II, l at -60 mv in ASW is 0.035–0.06 msec^{-1},

depending on the value of κ selected, and somewhat larger at -100 mv. In 440K the effect of hyperpolarization on dissociation rate is much more appreciable. At -60 mv, l is approximately 0.2 msec^{-1}, and at -100 mv it is 0.4–0.55 msec^{-1}. The significance of the larger V effect in high external K$^+$ is discussed below. (d) Finally, the value of l appropriate to C_9 is about 20 times less than the value used for C_5.

All of the other possibilities for QA–activation gate interaction that I could think of were considered and discarded. First, all models that lack something

TABLE II

CONSTANTS THAT GIVE REASONABLE FITS

Conditions	α	β	γ	δ	κ	l	$\bar{I}_K$*
	$msec^{-1}$	$msec^{-1}$	$msec^{-1}$	$msec^{-1}$	$msec^{-1}$	$msec^{-1}$	ma/cm^3
-60 mv, ASW	0.039	0.0911	0.01	0.1	0.35	0.05	3.9‡
	0.039	0.0911	0.01	0.09	0.17	0.035	3.9
	0	0.0925	0.05	0.08	0.30	0.06	3.0§
-100 mv, ASW	0	0.243	0.06	0.002	0.35	0.09	3.9‡
	0	0.243	0.055	0.002	0.17	0.07	3.9
	0	0.243	0.055	0.002	0	0.06	3.9
-60 mv, 440K	0.039	0.0911	0.01	0.1	0.35	0.22	2.9‡
	0.039	0.0911	0.01	0.09	0.17	0.18	2.9
-100 mv, 440K	0	0.243	0.06	0.002	0.35	0.55	2.9‡
	0	0.243	0.06	0.002	0.17	0.47	2.9
	0	0.243	0.06	0.002	0	0.40	2.9
$+90$ mv, ASW	1.1	0	0	0	0.63	0.03	3.9‡
	1.4	0	0	0	0.50	0.025	3.0‖
$+90$ mv, 440K	1.1	0	0	0	0.63	0.03	2.9‡

* $\bar{I}_K$ is the current density at $V = +90$ mv with all channels conducting.

‡ Calculations with these sets of constants are illustrated in Fig. 10.

§ For this recovery calculation, the test pulse had the constants marked by‖.

equivalent to q, the closed-inactivated state, can be rejected. Ready closing of the activation gates of occluded channels must be rejected because of the delayed fall of g_K in the presence of QA ions. Can QA ions escape from channels with closed n^4 gates? That is, is there a direct path from q to closed?

$$\text{closed} \rightleftarrows \text{open}$$
$$\uparrow \qquad \uparrow\downarrow$$
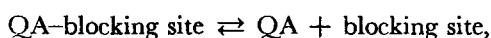$$\text{closed-inactivated} \rightleftarrows \text{inactivated}$$
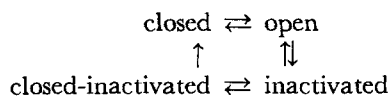
There are several objections to this scheme. For one thing, it has been shown that a channel must open before it can inactivate; i.e. the transition closed → closed-inactivated does not occur, so it would be necessary to suppose that the reaction could occur only in the direction closed-inactivated → closed, which seems unlikely. Further, this scheme explains neither the retarded recovery of

g_K at -100, nor its delayed fall. For similar reasons a direct path from inactivated to closed is also out of the question.

Is the Blocking Site in the Channel?

Two observations show that the blocking site occupied by QA ions is directly in the K^+ channel. The first is that increasing external K^+ increases l, the rate constant of dissociation of QA ions from the blocking sites. This means that dissociation of the QA–blocking site complex cannot occur altogether by the reaction

$$\text{QA–blocking site} \rightleftarrows \text{QA} + \text{blocking site,}$$

but that the reaction

$$K^+ + \text{QA–blocking site} \rightleftarrows \text{QA} + K^+\text{–blocking site}$$
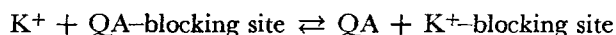
must also occur to a significant extent. If dissociation took place only by way of the second reaction, the rate of the reaction should be proportional to external K^+ with a proportionality constant of one. This does not seem to be the case, since l increases only by a factor of four or five when external K^+ is changed from a nominal 10 mm (actual concentration may be higher because of accumulation in the Schwann cell space) to 440 mm. Thus the second reaction seems to occur, but it is probably not the only dissociation reaction. The fact that the blocking site, which is accessible only to QA ions in the axoplasm, is subject to bombardment by external K^+ ions must mean that the blocking site is in the path of K^+ ions moving inward through the membrane, i.e., that it is in the K^+ channel.

The other observation supporting this idea is that hyperpolarization speeds dissociation. This cannot be a direct effect of the membrane field on the positively charged QA ion, because the effect is small when external K^+, is low. These facts can be explained by postulating that only a small fraction of K^+ ions normally have enough energy to displace a QA ion from a blocking site, but that K^+ ions gain energy in moving through the membrane field if V is negative, and thus dissociation is speeded. Both parts of the postulate seem reasonable. The rate of ion passage through an open channel is many orders of magnitude faster than the dissociation rate; if all had the energy to displace a QA ion the reaction would occur much more rapidly than, in fact, it does.

It also seems reasonable that K^+ ions can gain energy from the membrane field, and that a portion of this energy might not be dissipated through friction. The observed increase in l could well be accounted for in this way. Increasing the energy of a K^+ ion at the blocking site by 25 mev would in theory increase the reaction rate e-fold. The observed effect, approximately doubling the rate for a 40 mv change of V, is well within this theoretical limit. One could

equally well say that, for negative V, the K^+ concentration is increased in the membrane just external to the blocking site.

Pore or Carrier?

The fact that dissociation of QA ions from their blocking sites is speeded by hyperpolarization and by raising the external potassium ion concentration strongly suggests that K^+ ions traverse the membrane by means of pores, and equally strongly speaks against a carrier mechanism for passive K^+ movement. The effects of both V and external K^+ can be explained by supposing that K^+ ions move through pores and that each pore has near its inner end a site to which a QA ion can bind. If so, a sufficiently energetic K^+ ion moving inward through a pore could displace a QA ion from its blocking site, and the rate at which sufficiently energetic K^+ ions entered the pores would be proportional to the external potassium ion concentration. Further, a K^+ ion that lacked sufficient energy for QA displacement when it entered a pore could gain energy from the membrane field, and the energy so gained would be greater when the membrane is hyperpolarized. Neither the effect of V or of external K^+ on dissociation rate can be explained by a simple carrier model. (See, for examples, Horowicz et al., 1968; Adrian, 1969; Ciani et al., 1969.) By a simple carrier I mean a lipid-soluble substance that can complex to a K^+ ion, hydrated or dehydrated, at any point on either surface of the membrane, and that moves along its electrochemical gradient in a homogeneous region of the membrane. Such a carrier could in principle be inactivated by binding to a molecule that reduced its mobility or destroyed its ability to complex K^+ ions, but such models fail to explain many aspects of QA action. A difficulty for all carrier models, regardless of charge, is the necessity of explaining the asymmetry of the QA effect, i.e., the fact that QA ions block only when inside the (squid) axon. Why should a carrier that can bind K^+ ions at either surface of the membrane be able to complex with QA ions only at the inner surface? It cannot be said that QA ions cannot reach the outer surface of the membrane, for the experiments with external C_9 and C_{12} show that they do.

The V and K^+ effects cannot be explained regardless of the charge one selects for a model carrier. If the carrier-QA complex were positively charged, hyperpolarizing the membrane (making the inside more negative) would promote accumulation of the complexes at the inner surface of the membrane, and it is very difficult to see how *external* K^+ could, under these circumstances, be involved in the carrier-QA dissociation reaction. If the carrier-QA complex were neutral, V would not affect the location of the complexes and the effect of V on recovery rate could not be explained. If the complex had a net negative charge, hyperpolarization would tend to bring the QA-laden carriers to the outer edge of the membrane, where they would be exposed to external K^+ ion. There are, however, two serious problems. First, the uncomplexed carrier

would necessarily have a charge of -2, and would be immobilized at the inner surface of the membrane for large depolarizations, causing a marked decline of g_K. This does not, in fact, happen. Further, this model would require that QA-carrier dissociation take place at the outer membrane surface, even though formation of the complex could occur only at the inner surface. This would mean that the carrier could transport QA ions against an electrochemical gradient.

My conclusion is that simple carrier models cannot explain the data of this paper, regardless of the charge postulated for the carrier molecule. Though perhaps superfluous at this point, there are specific arguments against a carrier of nonactin or valinomycin type. These compounds are known to increase the K^+ permeability of lipid bilayers (Mueller and Rudin, 1967; Lev and Buzhinsky, 1967; Andreoli et al., 1967). It has been established by X-ray crystallography that nonactin folds to form a good fit for a dehydrated K^+ ion (crystal radius 1.33 A), with eight carbonyl oxygens surrounding the K^+ ion (Kilbourn et al., 1967). The crystallographic evidence thus strongly suggests that nonactin acts as a carrier, and the electrical evidence is compatible with this (Szabo et al., 1969). There are no data on the effect of TEA^+ or its derivatives on nonactin-mediated K^+ transport, but it seems safe to predict that the effect will be small, simply because the nonactin cavity is not large enough to accommodate TEA^+, which has a radius of about 4.5 A.

The available data also suggest that the number of K^+ ions transported by a nonactin molecule in a second is much smaller than the rate of K^+ movement in a K^+ channel or of Na^+ in an Na^+ channel. Pressman et al. (1967) estimate that each nonactin carries several hundred ions per second through mitochondrial membrane, a number that seems reasonable in light of two limitations on transport rate. These limitations are diffusion through the bilayer, which, according to Stein (1968), would limit the rate to a number of the order of one ion per millisecond and the rate of dissociation of the K^+-nonactin complex, which, at least in methanol, is estimated by Winkler (1969) to be 2×10^3 sec^{-1}. All three numbers are far smaller than the approximately 10^6 ions/sec estimated for K^+ channels (Armstrong, 1966) or the approximately 10^8 ions/sec estimated for Na channels (Hille, 1970).

Channel Specificity

The fact that the blocking site is in the channel is of great interest, for if one can determine the factors which allow, for example, TEA^+ to enter the channel, it will undoubtedly help in understanding why K^+ can enter the channels while Na^+ and other ions cannot. Some suggestions can be made on the basis of what is now known. TEA^+ has very nearly the same radius as a K^+ ion with one hydration shell (both are about 4.5 A), and since TEA^+ can enter the channel, there must be at least a portion of the channel that is able to accept

a K$^+$ ion with a complete hydration shell. C$_9$, which has a triethyl-N group, can also enter, but the trimethyloctylammonium ion, which lacks such a group, cannot. It is obvious that the radius of gyration of the QA ion is not very important, and it seems instead that the ion can block if the nitrogen-containing group retains a sufficient resemblance to TEA$^+$, or to a hydrated K$^+$. It will be of interest to see how other alterations of the groups around the nitrogen affect ability to enter the channel.

A possibility worth serious consideration is that K$^+$ ions lose their hydration shells after passing the site which QA ions can occupy, and that TEA$^+$, for example, cannot pass through the channel because its ethyl groups are not detachable. As pointed out by Mullins (1959) dehydration of K$^+$ ions can occur at a significant rate only if the membrane has some efficient means of lowering the large energy requirement for dehydration. A means for efficient dehydration of cations is contained in a model recently proposed by Urry (1971) to account for the facilitation of passive ion transport by gramicidin. Gramicidin lowers the resistance of thin lipid membranes, and Urry proposes that it does so by forming helical channels that are able to accept a dehydrated Na$^+$ or K$^+$ ion. According to this model, two helical gramicidin molecules transiently combine head-to-head to form a tunnel through the membrane that has a predominantly hydrophobic exterior, and a hydrophilic interior that is lined with carbonyl groups, capable of replacing the water molecules surrounding, for example, a K$^+$ ion.

The impermeability of QA ions and many aspects of their action on g_K are easily explained if one imagines that the outer part of a K$^+$ channel of a squid axon is a gramicidin-like tunnel with a large mouth at its inner end. The mouth is capable of accepting a hydrated K$^+$ ion or a QA ion, but the tunnel can accept only dehydrated K$^+$ ions, and QA ions are too large to enter. If this is so, it may be that two factors are involved in determining the selectivity of a a K$^+$ channel. The first would be the ease with which an ion enters the mouth of a channel, and the second factor would be the ease with which it enters the tunnel.

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